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Microbial interaction with diazinon, an organophospate pesticide.

Phyllis Joan Kuhn
University of Massachusetts Amherst

MICROBIAL INTERACTION WITH DIAZINON, AN ORGANOPHOSPHATE PESTICIDE

A Dissertation Presented

By

Phyllis Joan Kuhn

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Approved as to style and content by:

Hans B. Gunner
(Chairman of Committee)

R. E. Lewis

Warren Libby

August 1970

To

my husband,

Donald Edward Kuhn

and

my mother

Mrs. Phyllis Wall

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INTRODUCTION

The development of an effective pesticide which can be degraded in a limited period of time has become of paramount importance in recent years. Ever-increasing numbers of reports are appearing on the dangers of pesticides which are non-degradable. These have served to heighten public interest and increase concern regarding the widely used recalcitrant compounds such as DDT. Although DDT presents no apparent immediate danger to humans, considerable evidence has been established for danger to lower animals. The present levels of DDT in humans is approximately 12 ppm. This is an increase of about 50% from the late 1950's. The simple fact is that, at present, there is no known way to halt or reverse the buildup of DDT except to stop applying it. Therefore, the trend is to seek either biological controls of a highly specific nature, or to develop degradable pesticides with nontoxic residues which do not accumulate, as DDT does, in the tissues of the body. Thus far, a number of compounds which are effective in insect control, and are believed to be biodegradable, have become available. These are the organophosphates, each of which has the general formula (RO_2) P (S or O) OX, where X is a phenolic or a pyrimidinyl compound, and R an alkyl group. The alkyl phosphates inhibit

cholinesterase, causing the buildup of acetyl choline which serves to transmit impulses from nerve to muscle (21). Trade names include Diazinon, Parathion, Malathion, and Thimet. A pyrimidinyl organophosphate, Diazinon, inhibits cholinesterase and possibly other esterases. This compound, a contact poison synthesized by Dr. H. Gysin in the Geigy Laboratory in Switzerland, has proved effective against household, soil, vegetable, forage and fruit insects, and is of only intermediate toxicity to humans (11). Toxic effects of accidental exposure generally occur from contact with skin, or inhalation, and manifest themselves as dizziness, dyspnea, fibrillation, and contraction of pupils. Symptoms are relieved by atropine therapy.

Diazinon is generally applied at the rate of 1 lb/acre, and leaves only 0.75 ppm as residue after 2 weeks (11). The complete mechanism of degradation of the organophosphate in soil is obscure; however, the initial hydrolysis can be caused by absorption to clay, acid pH, action of microbes, or heat-labile substances in the soil, and ultraviolet light. The rate of degradation is dependent on Diazinon concentration, soil type, temperature, and moisture content.

In soil, Diazinon is known to absorb to clays of the bentonite series. This makes the pesticide non-extractable and ineffective. The clay, montmorillonite, is made of rectangular cell units. Water can come between these units,

expand the lattices, and expose all surfaces. When the clay is dehydrated, the charged layers are brought close together, forming strong covalent bonds with the Diazinon molecule. Some soils contain clays which adsorb Diazinon on the surface and sides. The mechanism for adsorption is not defined. In soil, degradation depends not only on clay content, but on organic content as well. Increasing organic content is directly related to increasing adsorption (22).

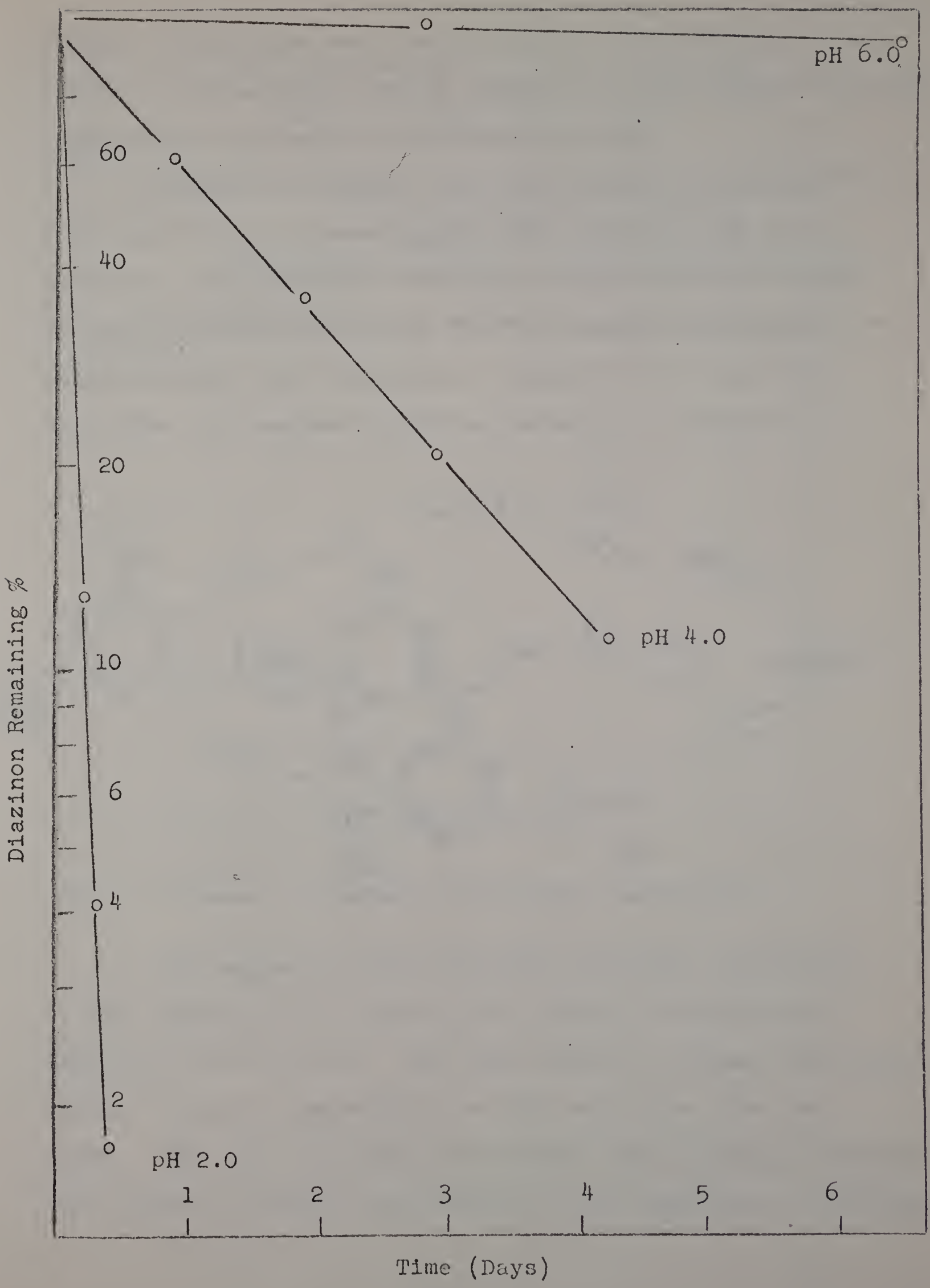
In an experiment by Konrad et al., in 1967, Diazinon degradation was traced in soil systems, and in acid systems without soil. Three Wisconsin soils, humic gley, gray-brown podzolic, and A C Prairie, with organic contents of 10%, 3.8%, and 1.6%, degraded Diazinon at rates of 11, 7, and 6% per day, respectively (22). A decrease in parent Diazinon and increase in degradation products was most apparent in humic gley than in the other soils tested.

Diazinon disappearance was determined by extracting samples with benzene, and measuring directly by gas chromatography, or by carbon 14 analysis. Diazinon was labeled in the 2 position of the pyrimidine ring, or the ethoxy side chain. The parent compound was extracted with benzene, and the breakdown products with butanol.

To test the effect of pH, Diazinon was added to an acidified aqueous solution. At neutral pH, Diazinon was very stable, with a half-life of 200 days, and the rate of

hydrolysis increased with decreasing pH. Diethyl thiophosphoric acid and 2-isopropyl-4-methyl-6-hydroxypyrimidine were the degradation products in soils and soil-free acid systems (Fig. 1). Two pathways for Diazinon degradation were proposed, and the distribution of hydrolysis products between different solvents was used to determine which pathway was valid. At pH 1.2, 16% of ring-labeled C^{14} was extracted with n-butanol, but when the pH was adjusted to 3.3 before extraction, 80% of the C^{14} activity was in the n-butanol fraction. This was identical to the percent radioactivity in a sample with a pH of 4.0, indicating the products formed at pH 1.2 and 4.0 were identical, and that the solubility was dependent on pH. The thiophosphoryl pyrimidine was ruled out as a hydrolysis product because the acidic thiophosphoryl group would be less soluble in butanol than in water. Therefore, the hydrolysis product was 2-isopropyl-4-methyl-6-hydroxypyrimidine. Protonation of the N in the pyrimidine ring increased the water solubility of the 2-isopropyl-4-methyl-6-hydroxypyrimidine, yielding a cation. Hydrolysis of the chain-labeled Diazinon produced, in contrast, 79% of C^{14} in the aqueous phase at pH 4, and 11% at pH 1.2. Adjustment of pH 1.2 to pH 3.3 yielded 71% in the aqueous phase. Again, similarity in distribution indicated that the products were the same. A decrease in the ionization of diethyl thiophosphoric acid at pH 1.2 increased the solubility in

Fig. 1.--Diazinon degradation correlated with pH. Data taken from Konrad *et al.* (9).


pH 6.0
pH 4.0
pH 2.0
60
40
20
10
6
4
2
0
1
2
3
4
5
6
Diazinon Remaining %
Time (Days)

butanol, explaining the distribution of radioactivity (Table 1). The major labeled product of chain-labeled Diazinon degradation was diethyl thiophosphoric acid.

At pH 1.2, benzene extraction yielded 11% of 14 C activity, but gas chromatography (GC) showed 0.14% intact Diazinon. The remaining radioactivity was probably caused by partial solubilization of ETOH in benzene from diethyl thiophosphoric acid hydrolysis. Comparison of soil and soil-free acid systems indicated pathway 1 a (Scheme 1).

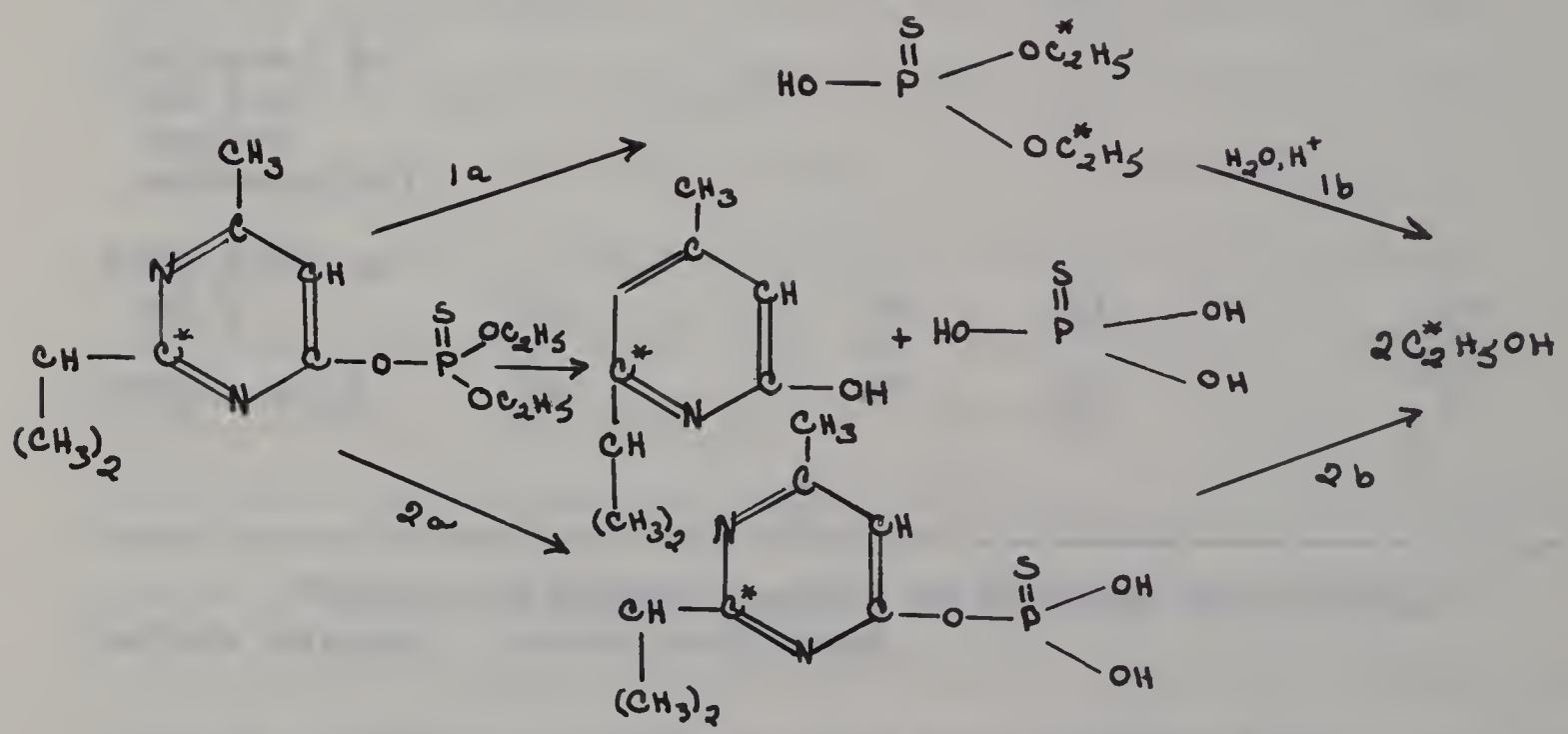


Fig. 2. Scheme 1.--Pathway of Diazinon degradation.

The degree to which microbial activity contributed to the degradation of Diazinon was tested by inoculating media with soil extract. The soil extract in these particular samples showed no degradation of the pesticide after two weeks. This led the author to conclude that microbial degradation was not a factor contributing to the breakdown of Diazinon

TABLE 1.--Distribution between n-butanol and water (1:1) of Diazinon hydrolysis products formed in soil-free aqueous solutions and degradation products formed in a Poygan sicl system

	C^{14} Distribution of Diazinon Degradation Products*			
	Ring-labeled Diazinon		Chain-labeled Diazinon	
	Butanol	Water	Butanol	Water
Soil-free at pH 1.2	16	84	89	11
Soil-free at pH 1.2 (Adjusted to pH 3.3 before extraction)	80	20	29	71
Soil-free at pH 4	80	20	21	79
Poygan sicl	80	20	23	77

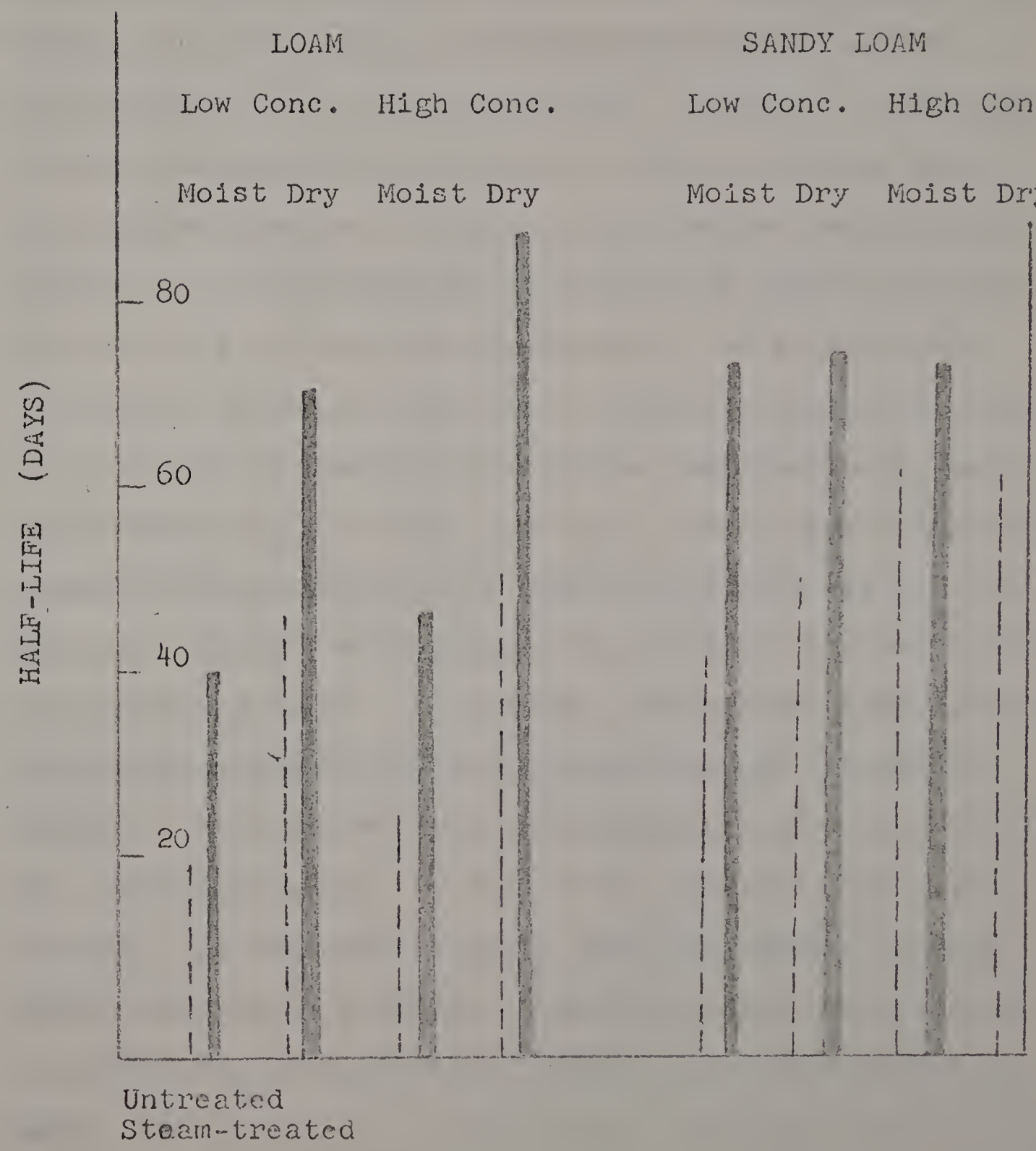
*Parent Diazinon removed by benzene extraction before butanol: water partition.

in soils, and that degradation was caused by the chemical process of hydrolysis.

Five months after this publication, Bro-Rasmussen and co-workers at the National Pesticide Laboratory in Copenhagen related Diazinon disappearance to 4 factors--concentration, moisture, soil type, and microbial activity (5). The greatest effect on Diazinon disappearance was microbial activity, and the least, Diazinon concentration (Fig. 3). Studies were also performed on various combinations of these factors. To rate the microbial activity factor, the authors employed a control soil treated with steam at 100°C for 1 hour to kill the majority of micro-organisms. Soil samples, carefully mixed with Diazinon granules, were moistened with the appropriate concentrations of water, and analyzed at 0-96 days. Samples were extracted with petroleum ether, evaporated, chromatographed, eluted, and analyzed by GC. The most dramatic difference was between steamed and untreated soils. Equally significant was the effect of changing the water capacity from 25 to 75%. Untreated soils and moist soils degraded Diazinon faster than steam-treated and dry soils. Decreasing Diazinon concentration was correlated with an increasing rate of degradation.

The difference in the experimental design of the Bro-Rasmussen and Getzin experiments led the authors to

Fig. 3.--Half lives of Diazinon in soil correlated with soil type, moisture content, sterilization and Diazinon concentration.
Data taken from Bro-Rasmussen *et al*. (2).


LOAM
Low Conc.
High Conc.
Moist
Dry
Moist
Dry
SANDY LOAM
Low Conc.
High Conc
Moist
Dry
Moist
Dry
HALF-LIFE (DAYS)
80
60
40
20
Untreated
Steam-treated

differing conclusions; according to Bro-Rasmussen, micro-organisms play an important role in degradation, whereas according to Getzin, they play no role whatsoever. The experimental approach of each leaves something to be desired. Bro-Rasmussen had no control for pH. Thus, in comparing results in steam-treated soil with those in untreated soil, the acidity produced by microbial activity in the untreated soil is not considered. Getzin, in studying Diazinon degradation in the soil, removed microbes from the soil, placed them in a liquid culture medium under aerobic conditions, and discounted the hydrolytic capacity of the soil, and the action of the anaerobes. In a later publication by Getzin in 1967 (12), comparing degradation products of labeled Diazinon in sterile fumigated soil, and non-fumigated soil, he did, in fact, report more $C^{14}O_2$ released from non-sterile soil (34%), than from sterile soil (0%), and slightly more hydrolysis products in sterile than in non-sterile soils. In summary, the primary hydrolysis to 2-isopropyl-4-methyl-6-hydroxypyrimidine and diethylthiophosphoric acid can occur non-biologically, and in a sterile soil, one would expect to find these products, and parent Diazinon. In non-sterile soil, one would expect to find primary hydrolysis products, parent Diazinon, and evolution of labeled CO_2 as evidence of disruption of the pyrimidine ring.

Getzin also found that the rate of degradation in non-sterile soil was correlated with temperature, and that there was an eight-fold decrease in the time required for a 50% loss from 15 to 35° (Fig. 3) (13). In some instances, the means of sterilization have also been correlated with the degree of organophosphate degradation. An experiment designed to test the effects of different sterilization methods on pesticide degradation was performed by sterilizing soils with gamma radiation from a cobalt source, and by autoclaving for 20 min at 15 psi (Table 2). Degradation of Malathion, Zinophos, and Diazinon were compared in non-sterile soil, and sterile soil which had been irradiated, autoclaved, or irradiated and autoclaved. Cobalt 60 was the radiation source, and the samples received 4-mrad doses at the rate of 250,000 rads per hour. Malathion degradation was equal in the non-sterile and irradiation-sterilized samples. The rate of degradation was greater than in the autoclaved samples. Zinophos degradation was greatest in the non-autoclaved samples and least in the autoclaved samples. The percent Diazinon degradation was the same in all four samples (14).

A heat-labile substance which degraded Malathion, an organophosphate less stable than Diazinon, was extracted from 4 of 5 soil samples from western Washington. The origin and nature of the heat-labile substance remains under

Fig. 4.--Diazinon degradation correlated with temperature in non-sterile soil.
Data taken from Getzin (5).

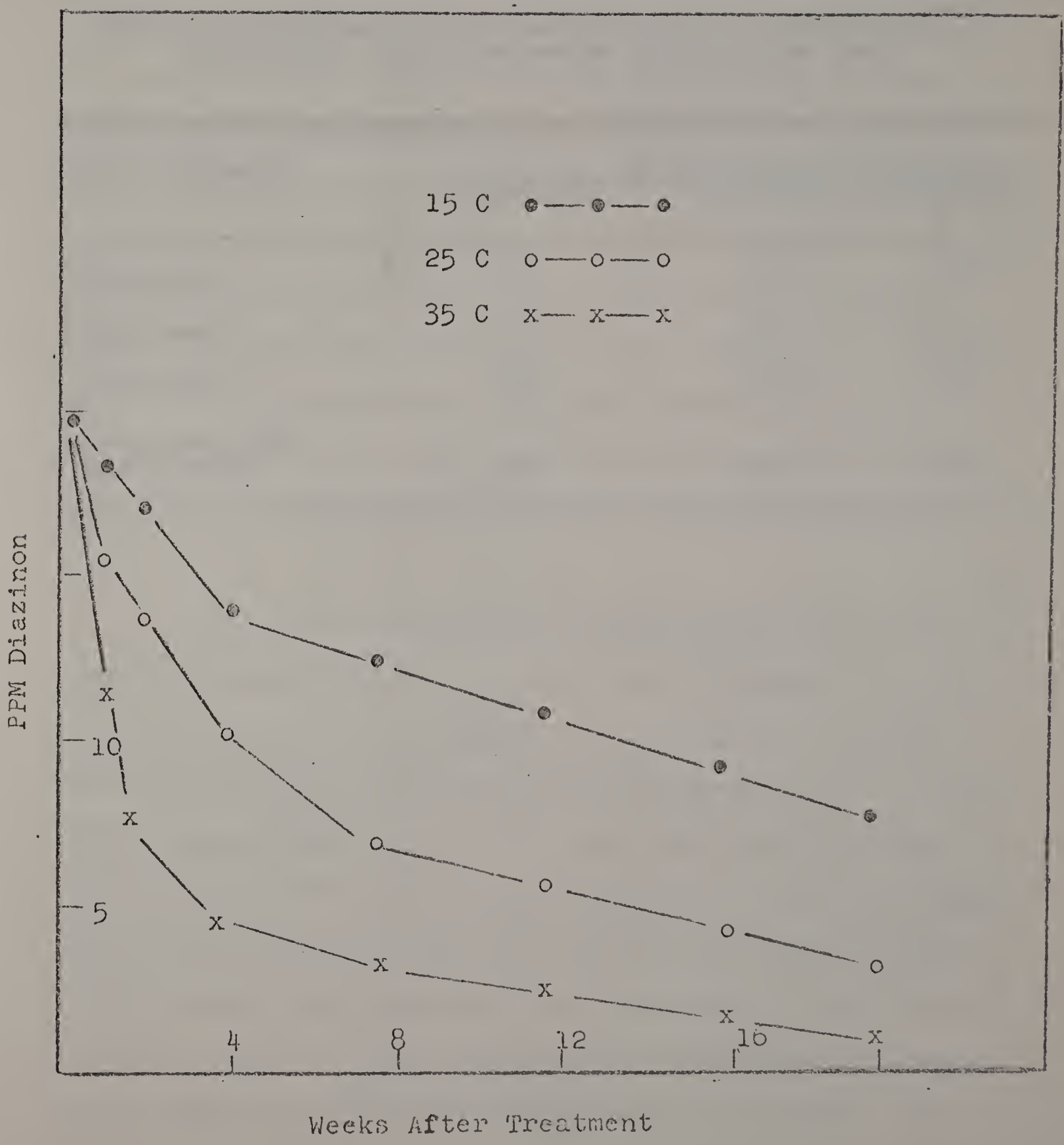

15 C
25 C
35 C
PPM Diazinon
10
5
4
8
12
16
Weeks After Treatment

TABLE 2.--Degradation of insecticides in irradiated, autoclaved, and non-sterile Sultan silt loam

Soil Treatment	% Insecticide Degraded		
	Malathion	Zinophos	Diazinon
Nonsterile	99	68	45
Autoclaved	11	23	42
Irradiated	98	31	44
Irradiated and Autoclaved	10	22	44

investigation. One explanation offered is that this is a heat-labile enzyme(s). Though irradiated microbes cannot multiply, many enzymatic activities still continue, and enzymatic fractions which degrade Malathion have been isolated (14). The fraction isolated from soil was stable for 3 months after irradiation, suggesting that it may represent a permanent component in the soil. Though, as mentioned earlier, 3 out of 4 soils in western Washington contained this fraction, 4 soil samples from the more arid eastern part of the state did not contain this material. These findings suggest that stringent controls are necessary in determining organophosphate degradation in soil, and that means other than heat-sterilization may be needed to determine the extent of microbial degradation in the soil.

The problem of possible interference from heat-labile substances in the soil may be circumvented by isolating the operative organisms by selective culture techniques and subsequently studying degradation in culture media rather than in the soil. One such study involved degradation of Diazinon by the combined action of an Arthrobacter sp. and Streptomyces sp. (15). The Arthrobacter sp. could degrade the side chain, as evidenced by the evolution of $C^{14}O_2$, when incubated with side chain-labeled Diazinon and buffer, but failed to degrade the ring moiety. The

Streptomyces sp. was also unable to degrade the ring. On mixing the 2 cultures together with ring-labeled Diazinon, Gunner and his co-workers found 15 to 20% of the label in $C^{14}O_2$ precipitated as $BaC^{14}O_3$, indicating synergistic attack on the ring. After 3 weeks incubation, most of the parent compound was converted to 2 unidentifiable metabolites.

Recently, Sethunathan and Mac Rae investigated the persistence of Diazinon in submerged soils, and found only 0.4-0.7% of the ring portion of Diazinon converted to CO_2 (30). Matsumura and Boush isolated a soil fungus, Trichoderma viride, which was "active" in the degradation of carbamates, possibly through a strong oxidative system (28). These same workers found a pseudomonad, a symbionte of the apple maggot, that degraded various pesticides through a strong oxidative system (27).

In a very early study by Ahmed and Casida, in 1958, a yeast, Torulopsis, the bacteria, Pseudomonas fluorescens and Thiobacillus thio-oxidans, and the algae, Chlorella, were tested for their ability to degrade organophosphates. Torulopsis and Chlorella hydrolyzed Thimet, and Am. Cyanamide 12008 and their derivatives. Certain derivatives were also oxidized. Pseudomonas and Thiobacillus hydrolyzed Thimet. An attempt to use Thimet as the sole sulfur source for Thiobacillus failed, but growth occurred after addition of elemental sulfur (1).

Thus, in reviewing the literature, it is apparent that little that is definite has been revealed about the pathways of microbial degradation of Diazinon, or if, in fact, that these even occur. Very few organisms have been implicated in Diazinon breakdown, although reports of the strongly selective effect of Diazinon on the soil microbial population have been reported. Of particular interest in this regard was the observation that although parent Diazinon was no longer present in a treated soil, the selective effects were sustained, presumably through the interaction of intermediates of Diazinon breakdown. It therefore emerges that biodegradation of the parent compound alone can no longer be equated with "innocuousness." In this light, the work reported in the following was undertaken to investigate in further detail the process of Diazinon degradation. Diazinon was chosen as representative of the organophosphates, a widely used class of presumably biodegradable pesticides. Further, in appreciation of the apparent ecosystem stress introduced among the microbiota by this chemical, it was also undertaken to examine the physiological response of microorganisms to this material.

MATERIALS AND METHODS

I. Cultivation of Organisms:

A. Growth Conditions:

1. Medium:

Cells were grown in modified Morris medium (MM).* The medium was altered by increasing the concentration of KH_2PO_4 from 0.30 to 0.32%, thereby decreasing the pH of the medium from pH 7.1 to pH 6.9. The phosphate buffer, composed of K_2HPO_4 and KH_2PO_4, glucose, and $FeCl_3$ were added separately, in that order, after the medium had cooled. This modification produced a clear, colorless solution. The medium was supplemented with 0.025% yeast extract for growth of auxotrophs. Stock cultures were maintained on nutrient agar stabs or on slants at room temperature.

2. Environment:

a. Aerobic:

Cultures were grown aerobically in 250 ml Erlenmeyer flasks, or in 150 ml test tubes filled with 50 ml of MM with 0.025% yeast extract, unless otherwise stated, or on nutrient agar plates.

b. Anaerobic:

Cultures were grown anaerobically in 16 x 75 mm

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*Appendix I.

screw-capped tubes filled with MM and 0.25% yeast extract, unless otherwise stated, that had been freshly autoclaved, and quickly cooled to minimize the amount of dissolved oxygen. Cultures were also maintained in partially filled Erlenmeyer flasks, or on nutrient agar plates under a carbon dioxide atmosphere in a vacuum incubator (Jas Marsh Co.) or in 150 ml test tubes sparged with nitrogen. Most cultures were grown at room temperature.

3. Measurement of Growth:

The increase in cell mass was measured as a change in optical density at 620 nm in a Spectronic 20 colorimeter (Bausch and Lomb). New, unscratched screw-capped tubes served simultaneously as cuvettes, and culture tubes. A Klett Summerson Photoelectric Colorimeter with a red filter (maximum transmission 600 nm) was used in preliminary experiments. Nephlometer flasks from Bellco Glass, Inc. or Erlenmeyer flasks with bent glass side arms, 4.5 cm from the base, 1.5 cm wide, and 17 cm long, were used in replacement cultures. The side arm served as the cuvette.

B. Isolation of Organisms:

1. Anaerobes:

Enrichment cultures were prepared in 25 ml screw-capped tubes filled with MM, Diazinon, 0.13% (reagent grade City Chemical Co., New York); and glucose, 1.5% Diazinon; or Diazinon, 0.13%, yeast extract, 0.5% and casamino

acids, 0.3%, as carbon sources. These were inoculated with approximately 1 g of soil or mud. Parallel control cultures omitting Diazinon were also prepared and inoculated from the same source as the corresponding test cultures with Diazinon. All tubes were incubated 1 week at 30 C.

After incubation, 0.1 aliquots of each sample were diluted to 10^5, 10^6, and 10^7, and spread on nutrient agar plates. The plates were incubated anaerobically at 30 C under a CO_2 atmosphere. Various colonies were isolated from these plates. The isolates were restreaked on nutrient agar plates, incubated as previously described, and kept as stocks in stab cultures. All isolates, with the 1 exception, were facultative anaerobes.

2. Aerobes:

Aerobic enrichment cultures were prepared by inoculating dilution bottles containing 10 ml of water and 0.1 ml Diazinon, or 10 ml of water containing 0.025% yeast extract and 1% Diazinon with soil, mud, cow manure, water or sewage effluent. Cultures which became turbid after incubation at room temperature from 1-3 weeks were streaked on nutrient agar plates, and incubated 24-48 hours at 30 C. Isolates were restreaked on plates, incubated, and kept in stock on nutrient agar slants.

An Acetobacter sp. was isolated from vinegar cider on calcium carbonate-ethanol agar. The Acetobacter sp.

produced acid from the ethanol, which dissolved the calcium carbonate, and produced a clearing in the agar. The colonies were examined microscopically, and restreaked on ethanol agar. They were subsequently restreaked on agar made of MM with an acetate carbon source (32).

C. Characterization of Isolates:

A number of isolates were characterized by use of the gram stain, flagellar stain (Baily Method**), and India ink stain for capsules.** Cultures were examined in wet mount preparations under a phase microscope. A number of cultures were also examined by electron microscopy.

The organisms, D_{83} and AC, previously implicated in Diazinon degradation (16), were further characterized by their growth on Levine EMB agar*, gelatin*, litmus milk*, triple sugar iron agar*, and nutrient agar. The nutrient agar was examined under ultraviolet light for fluorescent pigments produced by the colonies. Colonies were also tested for the production of catalase by the application of 3% hydrogen peroxide and the appearance of oxygen bubbles in the solution.

D. Nutritional Requirements:

A nutritional study was undertaken using various

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*Appendix I.

**Appendix II.

combinations of 20 pooled amino acids* in 1.6% Noble agar and MM to characterize the isolates further.

E. Replacement Cultures:

Replacement cultures were grown aerobically in MM which was supplemented with Diazinon, solubilized in ethanol or Diazinon in concentrations of 1.8%, 1.5%, 0.3% and 0.026% as the respective sole phosphorus, carbon, nitrogen and sulfur sources. Cultures were initially grown in nephelometer culture flasks and shaken on a rotary shaker model G-52 (New Brunswick Inst.) at 28 C. When Diazinon was used in substrate amounts, an emulsion was produced which caused interference with optical density readings. This interference was eliminated by the use of stationary cultures with a large surface area. The cultures were shaken gently before reading to disperse the cells, and decanted into a bent side arm which served as a cuvette in which to siphon off the culture without disturbing the heavy Diazinon emulsion at the bottom of the flask.

F. Screening Technique:

Twenty aerobes and 16 anaerobes were tested for their ability to use Diazinon as a sole source of carbon or sulfur. Plates were prepared with MM, 1.5% Noble agar, and 0.1 ml of Diazinon as the sulfur or carbon source. Diazinon was spread on the agar surface with a sterile glass rod. The

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*Appendix II.

plates were divided into 4 sections, and inoculated. Cultures were examined each day for 7 days, or until growth appeared. Once a culture grew, it was transferred on to a solid medium with Diazinon as the sulfur or carbon source. The culture was then passed through a series of transfers in liquid media, consisting of MM with Diazinon as the sulfur or carbon source.

G. Detection of P=S:

A very sensitive test is available for the detection of the phosphorus-sulfur bond in the Diazinon molecule (2). A solution of ammonium palladium chloride* is sprayed on a sample on a thin layer chromatography (TLC) plate. The spray produces a yellow color when it comes in contact with the P=S of the Diazinon molecule. This technique is sensitive to 1 µg.

TLC plates were prepared from Absorbosil (Applied Science Laboratories, Inc.), to a thickness of 250 µ and activated 1 hr at 125 C. Approximately 3 ml of the supernate, or the culture, which contained Diazinon as the sole sulfur source, was extracted with 1 ml of petroleum ether. The extract was allowed to evaporate to 0.1 ml, and spotted 3.5 cm from the bottom of the plate. The plates were developed in acetone-butanol (1:4 v/v) solution in a presaturated atmosphere in sealed glass tanks. When the

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*Appendix II.

solvent front was 2.5 cm from the top of the plate, the plates were removed and air dried. Ammonium palladium spray was applied, and allowed to develop for 24 hr for full color production. The higher the concentration of P=S the greater the color intensity.

II. Effects of Diazinon or Breakdown Products on Bacteria:

A. Effects of Diazinon on Generation Times:

The effects of Diazinon on generation times of aerobes and anaerobes was examined. Aerobic cultures were grown in MM supplemented with 0.025% yeast extract and anaerobic cultures in MM containing 0.5% yeast extract, and 0.3% casamino acids, respectively. Mycobacterium phlei was grown in 0.5% yeast extract. The test cultures contained 0.1% of Diazinon which insured saturation of the medium.

B. Effects of Breakdown Products on Generation Times:

Cultures of anaerobes 6, 7, and 9P were grown through two 24 hr transfers in MM supplemented with 0.025% yeast extract. Culture 9 was grown in unsupplemented MM. Growth rates of control cultures, and cultures supplemented with approximately 0.1% 2-isopropyl-4-methyl-6-hydroxypyrimidine, 0.1% of the ammonium salt of diethyl thiophosphoric acid, 0.1% ethanol, or 0.1% Diazinon were compared to determine the moiety most active in effecting growth rates. Since side chain and ethanol had similar effects on most cultures, an experiment was conducted to determine if changes in growth

rates varied directly with equivalent concentrations of diethyl thiophosphoric acid and ethanol.

Cultures were supplemented with ethanol, and diethyl thiophosphoric acid in amounts equivalent to 40 ppm, 102 ppm, 1502 ppm, and 31,000 ppm Diazinon.

C. Identification of 2-isopropyl-4-methyl-6-hydroxypyrimidine by Infrared Spectrophotometry:

An infrared (IR) spectrum was obtained on the 2-isopropyl-4-methyl-6-hydroxypyrimidine in a Nujol Mull on a Perkin Elmer 457 Grating Infrared Spectrophotometer.

D. Effects of Diazinon on Total Nucleic Acid Synthesis:

Samples (3 ml) of cultures 6, 7, 9, and 9P were taken at timed intervals, and centrifuged. The supernate was discarded, and the cell pellet washed in 2 ml of phosphate buffer, pH 7.0 and by 2 ml of cold 7% perchloric acid to remove nucleic acid pools. Cultures were centrifuged, and the supernate discarded. Aliquots (2.5 ml) of 7% perchloric acid were added to each sample. Samples were placed in a boiling water bath for 30 min, cooled, and recentrifuged. The supernate was transferred to a cuvette, and the optical density at 260 nm determined.

E. Effects of Diazinon and Breakdown Products of Cytology:

Phase contrast micrographs and electron micrographs of negative stains or of thin sections of treated cultures,[1]

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[1]Appreciation is expressed to B. Takacs for micrographs of negative stains and to S. C. Holt for micrographs of thin sections of *M. phlei* and a negative stain of *R. rubrum* M_2B.

and controls were prepared and examined.

III. Diazinon Degradation; Enzymatic or Nonenzymatic; Detection of Parent and Breakdown Products:

A. Assay by Gas Chromatography:

Diazinon was assayed quantitatively by electron capture gas chromatographh (10) (GLC) using a Varian Aerograph, Series 1200 instrument.

Column type: Glass

Column size: 1.5 M x 3 mm

Support: Chromasorb G

Mesh: 60-80

Coating: 6% QF-1

Column temperature: 200 C

Injector temperature: 250 C

Detector temperature: 220 C

Nitrogen flow: 27-30 ml/min

Glass injector liner

Recorder-chart speed: 1.25 cm/min

Diazinon had a retention time of 1.5 min. The method was sensitive to 1 nanogram/microliter.

1. Diazinon Degradation by Anaerobes:

Isolates 1, 2, 4, 7, 11, and 16 were grown in 50 ml of MM supplemented with 30 ppm Diazinon in 250 ml Erlenmeyer flasks in a carbon dioxide atmosphere. Small, 2 ml aliquots were extracted with petroleum ether (v/v), each day for 12

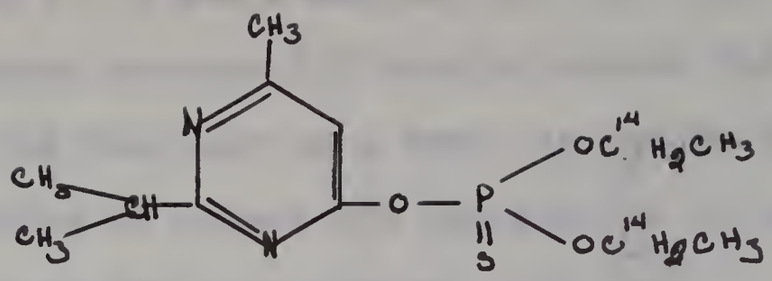
days, and 1 µl amounts injected into the gas chromatograph. The pH of the cultures was monitored on a Beckman Model 72 pH meter, with a combination electrode. Controls consisted of uninoculated media adjusted with hydrochloric acid to the pH the culture achieved after 24 hr incubation.

2. Diazinon Degradation Correlated with pH and Oxygen Tension:

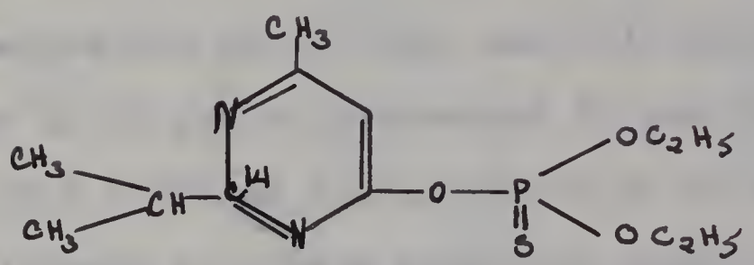
A facultative anaerobe 7 was grown in nutrient broth supplemented with 10 ppm Diazinon under aerobic, semi-aerobic, and anaerobic conditions. Erlenmeyer flasks (250 ml) contained 50 ml of medium for aerobic conditions, and 200 ml for semi-aerobic and anaerobic conditions. Anaerobic cultures were grown in a carbon dioxide atmosphere. The cultures' pH were determined and their Diazinon concentrations were assayed on each of 5 days by GLC. Uninoculated controls at the same pH as the culture were also prepared and assayed. The percent Diazinon degradation in each sample was determined and compared to a control.

B. Radioactive Tracer Techniques:

Detection and identification of Diazinon and Diazinon breakdown products were accomplished by using ring and side chain-labeled Diazinon obtained from the Geigy Chemical Corporation, Ardsley, N.Y.



Specific Activity 3.4 μc/mg
13.9 mg = 50 μc = 111 x 10^6 dpm (20)
1 μc = 3.7 x 10^4 dps



Specific Activity 3.6 μc/mg
14.7 mg = 50 μc = 111 x 10^6 dpm

Stocks were initially diluted with 25 ml of ethanol, and subsequently diluted 1:100 with ethanol. Theoretically 0.1 ml of ring or side chain was equivalent to 4,400 cpm, but in actual experiments, 0.1 ml of ring-labeled Diazinon was equivalent to 3000 cpm, and 0.1 ml of side chain to 1,500 cpm. These values decreased as the stocks aged, for Diazinon was slowly degrading with time.

1. Use of Labeled and Unlabeled Diazinon:

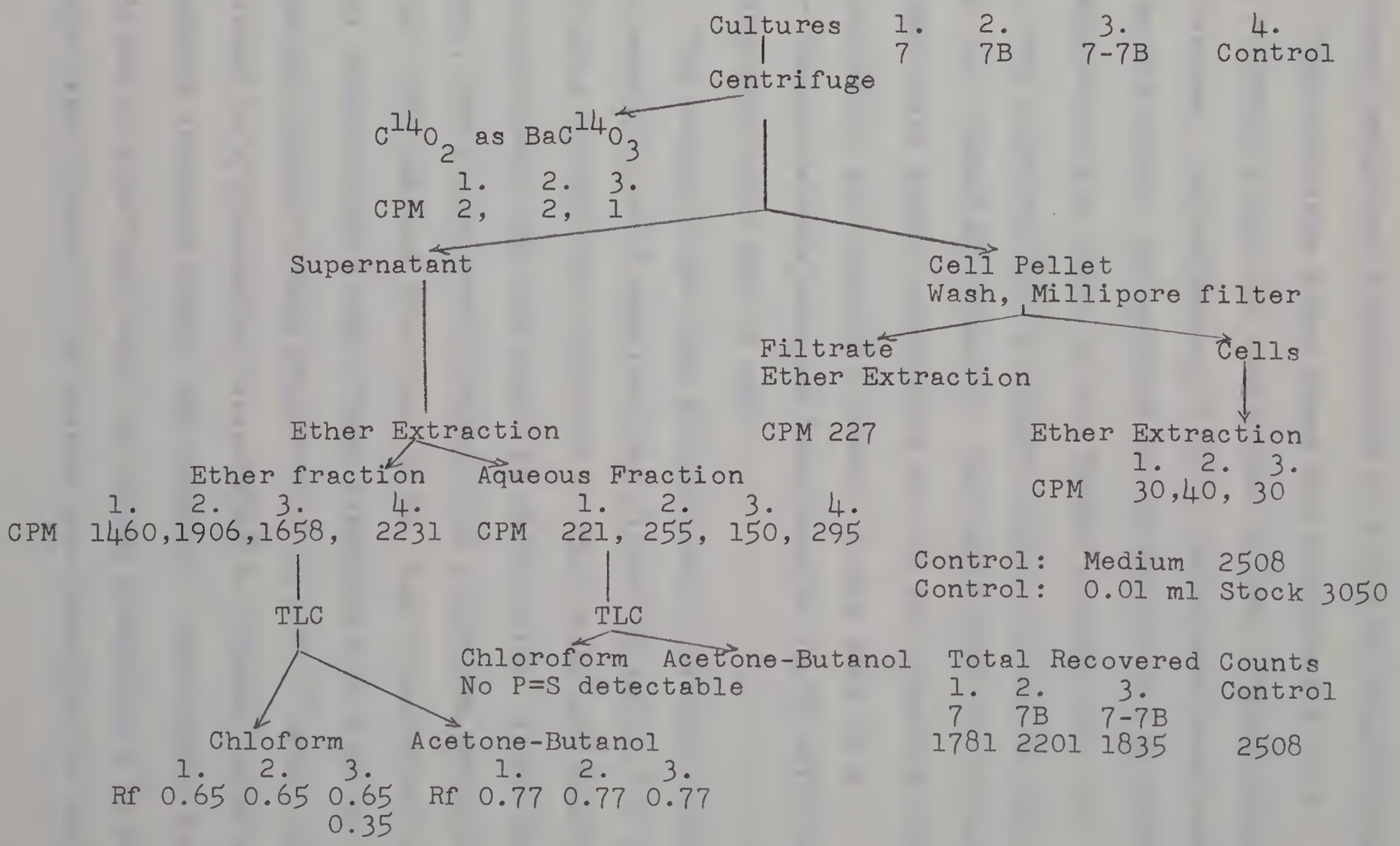
In a preliminary experiment, unlabeled and labeled Diazinon was added to the anaerobic cultures and controls. Diazinon degradation was monitored by counting the aqueous and petroleum ether fractions of the supernate and the cells, and by counting labeled CO_2 as Ba CO_3. Cultures examined

included 7, 7B and a mixture of 7 and 7B. These cultures were chosen because GLC results showed the culture 7, degraded 69% of the Diazinon in 4 days, as compared to 34% in a control and culture 7B caused 48% degradation of the Diazinon, as compared to 28% in a control (Fig. 29).

Morris medium (500 ml) was supplemented with 0.5% yeast extract, 0.3% casamino acids, ring-labeled Diazinon in a concentration of 500 cpm, and cold Diazinon in a concentration of 60 ppm as determined by gas chromatography. Test tubes (2.5 x 20 cm) containing 30 ml of medium were sparged with nitrogen to create anaerobic conditions. These tubes were inoculated with cultures 7, 7B, and a mixture of 7 and 7B. The effluent gas was bubbled through 45 ml of a saturated solution of barium hydroxide which precipitated the carbon dioxide as barium carbonate (Scheme 2).

After 120 hr at 25 C, the cultures were centrifuged at 10,000 rpm for 15 min. A 2 ml sample of supernate was extracted with 2 ml of petroleum ether, and the petroleum ether extract scanned on a Beckman DB-G Grating Spectrophotometer. The maximum absorption of Diazinon was at 240 nm. The peak height was compared to the initial peak height at zero time, and to a sterile control adjusted to the culture's pH. The petroleum ether extract and the aqueous phase were added back to the supernate, and the entire 30 ml supernate extracted with two 50 ml and one 30 ml

Fig. 5. Scheme 2.--Protocol for the study of microbial degradation of C^{14} ring-labeled and unlabeled Diazinon.



portions of petroleum ether in a 150 ml separatory funnel. The aqueous phase was transferred to a 250 ml round-bottom flask. The petroleum ether phase was transferred to a 250 ml Florence flask and evaporated to near dryness under an air stream. Approximately 2 ml of petroleum ether were added to the flask, and swirled to solubilize any Diazinon that adhered to the sides of the flask, reevaporated to 0.5 ml, and pipetted onto a ridged copper planchette (Nuclear Chicago Co., Cambridge, Mass.). Two additional ml of petroleum ether were added, evaporated to 0.5 ml, pipetted onto the planchette, and counted 5 times, 10 min each in a Nuclear Chicago windowless gas flow counter (0.1% efficient). The background count was 22 cpm.

The material was then solubilized in diethyl ether, divided equally into 2 samples, and placed on each of 2 thin layer plates coated with Absorbosil. One plate was developed in acetone-butanol and the other in chloroform. After development, the plates were sprayed with palladium ammonium chloride. The aqueous fraction, 30 ml, was transferred to a 150 ml round-bottom flask, flash evaporated to 2 ml at 11-12 mm Hg (Buchner Flash Evaporator) in a 50 C water bath, transferred to a planchette, evaporated to dryness under a Westinghouse Infrared light, and counted. The residue was solubilized in water and half the volume transferred to each of 2 thin layer plates. The plates were developed in acetone-

butanol, or in chloroform, dried, sprayed with ammonium palladium chloride, overlaid with x-ray film and examined after 10 days.

Controls consisted of radioactive, ring-labeled Diazinon (13.9 mg = 50 μc), and side-chain-labeled Diazinon (14.7 mg = 50 μc), which were diluted with 25 ml of ethanol, and cold Diazinon. The 0.01 ml samples of labeled Diazinon and 0.01 ml of cold Diazinon were spotted on thin layer plates, developed, sprayed with palladium ammonium chloride, and overlaid with x-ray film for 10 days. The film was subsequently developed and examined.

The cell pellet of each culture was resuspended in 150 ml of phosphate buffer. The cells were collected and washed on a membrane filter and the filtrate extracted with petroleum ether. The ether phase was evaporated to 2 ml, placed on a planchette, dried, and counted. The cells were rinsed off the membrane filter disc with 10 ml of phosphate buffer, extracted with petroleum ether, and the ether phase dried, and counted.

Carbon dioxide, collected as barium carbonate, was collected on 30 mm analytical filter paper (Schleicher and Schuell) by suction filtration. Samples were collected in 5 ml aliquots from the suspension of barium carbonate in the barium hydroxide solution. The filter papers were placed on flat planchettes previously coated with silicon grease to

prevent curling of the paper, and dried under infrared light. Samples were counted for radioactivity (15, 16).

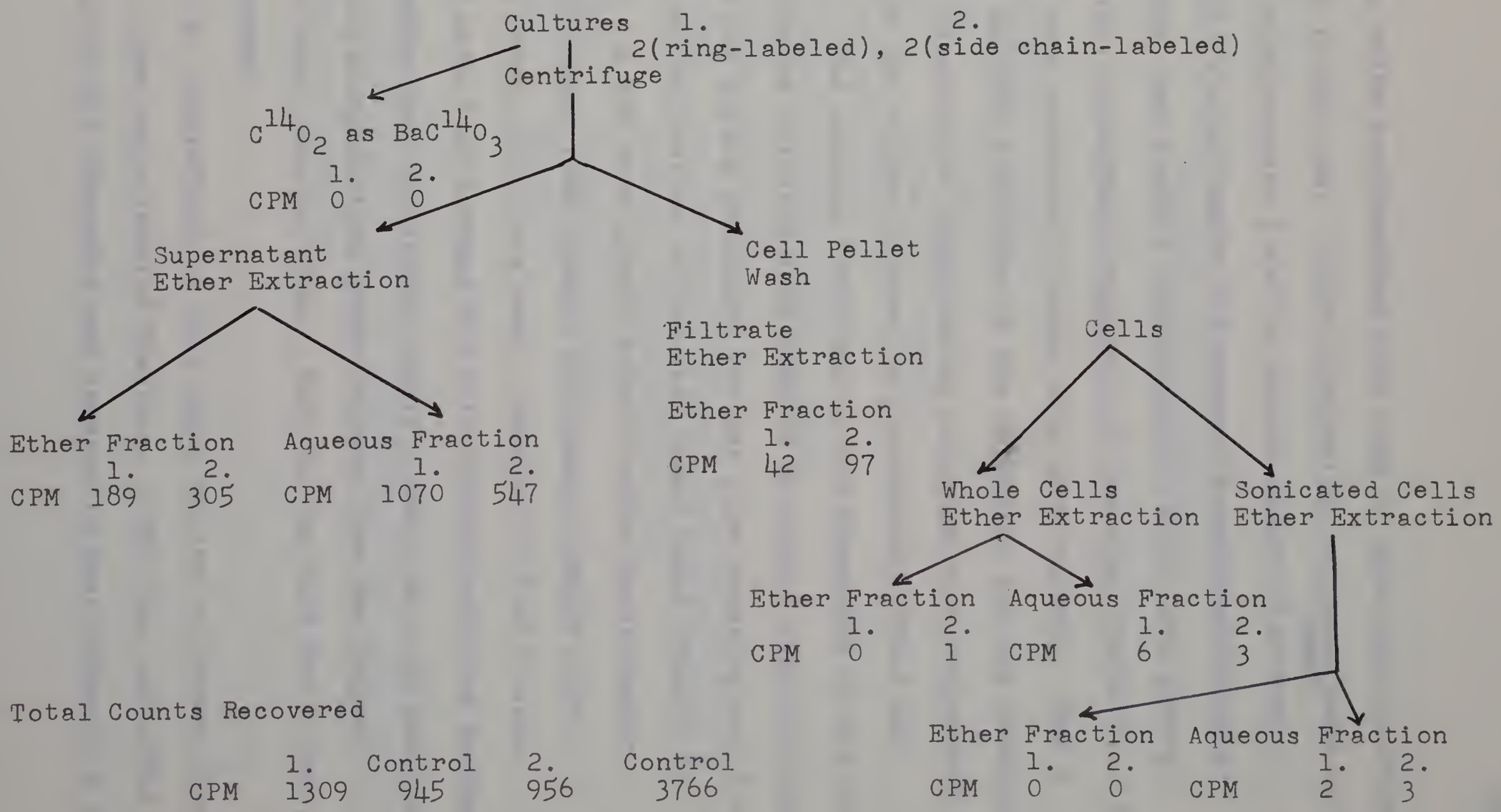
2. Use of Ring-labeled and Side Chain-labeled Diazinon:

In a second experiment, ring-labeled or side chain-labeled Diazinon in a concentration of 1000 cpm was added to a culture. The culture selected for this experiment was an anaerobic streptococcus 2, which degraded all the Diazinon, 25 ppm, in a culture in 3 days as determined by GLC. The design of this experiment was essentially similar to that of the previous experiment, but in addition to counting carbon dioxide, the ether and aqueous fractions of the supernate, the ether fraction of the cell wash, ether and aqueous fractions of sonicated and whole cells were examined (Scheme 3). The cells were rinsed off the filter paper disc with 10 ml of phosphate buffer, and divided into 2 5ml samples. Sample 1 was extracted with petroleum ether, and the ether and aqueous fractions evaporated and counted. Sample 2 was treated similarly, but was sonicated 15 min before extraction. A control of pH 5.1 was used to determine the total number of counts added to the sample.

3. Use of Solvent Partition to Determine Distribution of the Diazinon and Breakdown Products:

Anaerobes 1, 2, 7, and R. rubrum M_2B were incubated in screw-capped tubes 10 days in 0.5% yeast extract and 0.3%

Fig. 6. Scheme 3.--Protocol for the study of microbial degradation of C^{14} ring-labeled and side chain-labeled Diazinon



Total Counts Recovered

	1.	Control	2.	Control
CPM	1309	945	956	3766

casamino acids supplemented with ring or side chain-labeled Diazinon (1,000 cpm). After incubation, cultures were extracted with benzene (v/v) to remove parent Diazinon, butanol (v/v), to remove 2-isopropyl-4-methyl-6-hydroxy-pyrimidine, and diethyl thiophosphoric acid, and diethyl ether (v/v) to extract fragments of the ring (Table 3). All samples were extracted 3 times with each solvent. The 3 organic solvent fractions of the cultures, and the remaining aqueous fraction of ring-labeled cultures were evaporated to reduce the volume, placed on planchettes, dried and counted. The aqueous fractions of the supernate of the side chain-labeled cultures were counted by liquid scintillation because the drying procedure for gas flow counting would have caused volitalization of the side chain label present as ethanol. A Tri Carb Liquid Scintillation Spectrometer (60% efficient) from Packard Instrument Company, Inc., Downers Grove, Ill., was used. Samples (0.1 ml), were diluted with 1 ml Beckman Bio Solv Solubilizer from Beckman Instruments Inc., Fullerton, Calif., and 9 ml 0.4% Omnifluor in liquid scintillation grade toluene from New England Nuclear Corp., Boston, Mass., kept in the dark for 1 hour, and counted.

4. Conversion of Side Chain Moiety to Carbon Dioxide:

Side chain-labeled Diazinon, 1,000 cpm, was added to cultures of all anaerobes and aerobes in test tubes (2.5 x 20 cm).

TABLE 3.--Comparison of distribution of products in solvent partition systems.

An Aqueous Solution of Diazinon

Petroleum Ether - Water

Ether: Diazinon

Water: Hydrolysis Products

Sequential Extraction:

Benzene-Butanol-Diethyl Ether - Water

Benzene: Diazinon

Butanol: 2-isopropyl-4-methyl-6-hydroxypyrimidine

Diethyl thiophosphoric acid

Ether: Fragments of Ring

Water: Ethanol*

*Ethanol also extracted in the benzene, butanol, and ether fractions, but does not interfere with estimations of other products since it volatilizes during the preparation of samples for counting.

Nitrogen or air was continuously bubbled through the cultures to purge them of CO_2 produced by microbial metabolism. The effluent gas was bubbled through a solution of saturated barium hydroxide which precipitated the CO_2 as barium carbonate. The barium carbonate was collected, dried, and counted.

5. Use of Ring-labeled Diazinon and Cell Extracts to Investigate Enzymatic Hydrolysis of Diazinon:

An anaerobic culture no. 7 was grown in 500 ml of yeast extract and casamino acids. The cells were harvested, washed in glass distilled water, placed in an ice bath, and sonicated 5 min in a Bronwill Biosonick from Blackstone Ultra Sonics. Microscopic examination indicated almost 100% breakage. The solution was frozen overnight, thawed, and divided into 2 equal samples, A and B. Fraction A was incubated with ring-labeled Diazinon for 5 min. A control containing water and labeled Diazinon was also run. Both samples were extracted sequentially with benzene and butanol. The separate fractions were evaporated to reduce the volume, placed on planchettes, dried and counted. The samples were redissolved in appropriate solvents and spotted on 2 thin-layer chromatography plates, 1 of which was developed in acetone-methanol, the other in chloroform, as previously described, and overlaid with x-ray film, which was developed after 4 days.

Fraction B was eluted from a column containing

Bio-Gel P 60, 50-150 mesh (wet), from the Bio Rad Laboratories in Richmond, Calif. Bio-Gel, a polyacrylamide gel was hydrated with 22 ml of water overnight before use. The hydrated bed volume was 18 ml/dry gram, and the exclusion limit was 60,000 molecular wet. The column, 43.7cm x 2.5 cm contained a coarse sintered glass disc in the bottom. The top of the gel was protected with a small disc of filter paper. The column eluate (3 ml) was collected automatically every 3 min in a Radi Rac Fraction Collector (L.K.B., Stockholm, Swed.). Void volume, as determined by eluting 1 drop of blue dextran through the column, was 44 ml.

The optical density of the fractions was determined at 260 and 280 nm in silica cells with a 1 cm light path in a Beckman DBG, and mg protein/cc calculated (8). Fractions 1 through 15 were incubated with 0.1 ml ring-labeled Diazinon. Fraction 7 was divided into 2 equal amounts, and 1 fraction was boiled for 20 min. Scanning spectra in the ultraviolet region was performed on fraction 7. Samples were extracted with benzene, evaporated, placed on planchettes and counted.

6. Hydrolysis of Diazinon by Nucleic Acids:

Culture 7 was grown in 0.025% yeast extract and MM. The cells were collected by centrifugation, were washed in buffer, recentrifuged and the supernate discarded. Nucleic acids were extracted by boiling the cells 30 min with 7% perchloric acid. The suspension was centrifuged to remove

the protein, and the supernate, which contained the total nucleic acids, was saved. A perchloric acid control, and the perchloric acid-nucleic acid mixture were adjusted to pH 7 with sodium hydroxide. Samples were incubated with 100 ng Diazinon, extracted with 0.5 ml petroleum ether, and 0.1 µl of the petroleum ether extract was injected into the gas chromatograph.

7. Hydrolysis of Diazinon by Amino Acids:

Stocks of amino acids were adjusted to neutrality with sodium hydroxide, incubated with 0.1 ml of ring-labeled Diazinon (1000 cpm) for 5 min, extracted with benzene, and counted.

8. Hydrolysis of Diazinon by Purified Enzymes:

a. Deoxyribonuclease:

Amorphous deoxyribonuclease from beef pancrease, 2,000 kunitz units/mg, from Nutritional Biochemical Corporation in Cleveland, Ohio was an endonuclease which split DNA into 5' phosphate terminated polynucleotides (23, 24, 35). The enzyme was diluted to 0.1%. Diazinon, approximately 10 ppm, in 2 ml 1M acetate buffer pH 5, and 1 ml 0.1 M $MgSo_4$ served as the blank. A test was performed in a similar manner with 0.002 mg of enzyme. After a 30 min incubation, the blank and control were extracted v/v with petroleum ether, and 1 ml amounts injected into the gas chromatograph.

b. Alkaline Phosphatase:

Alkaline Phosphatase, a non-specific phosphomonoesterase, catalyzed the reaction:

Orthophosphoric monoester + water → alcohol and H_3PO_4

Alkaline phosphatase from calf mucosa (type 3) prepared by the Sigma Chemical Company in St. Louis, Mo., was diluted to 0.1% in 0.1M Tris buffer pH 8.5. A standard substrate of 0.00365 M Worthington carboxyl phenyl phosphate was diluted with 2 ml 0.2M glycine buffer, and 0.5 ml 0.05M $MgCl_2$ at 25 C/30 min. At zero time, 0.1 ml of enzyme was added to the test cuvette, and absorbancy of the hydrolysis product, salicylic acid, was recorded continuously on a Beckman DB-G Recording Spectrophotometer against a blank containing substrate, buffer and $MgCl_2$ (4, 19, 35). Specific activity was 0.4 units/mg.

In the test sample, 10 ppm Diazinon was added to the cuvette instead of standard substrate, and any change in the spectral scan from 240 to 270 nm, the Diazinon absorption, was noted. A water blank was used. The test sample and the control were also extracted with petroleum ether, and gas chromatographed as previously described.

c. Acid Phospatase:

Acid Phosphatase, a non-specific phosphomonoesterase with an optimal pH below 7, from Sigma Chemical Company in St. Louis, Mo., was diluted to a 0.01% solution in water.

Standard substrate, 0.00365 M Worthington o-carboxy phenyl-phosphate was added to each of 2 cuvettes in 0.5 ml amounts with 2 ml of 0.15 M sodium acetate buffer adjusted to pH 5 with HCL. Enzyme, 0.5 ml, was added to the test cuvette at zero time, and the absorbancy of the hydrolysis product, salicylic acid, recorded at 300 nm continuously on a Beckman DB-G. The activity was 0.13 units/ml (35).

$$\text{Units Activity} = \frac{\text{A/min x 1000}}{\text{3500 x mg/ml reaction time}}$$

One unit was equivalent to 1 micromole of o-carboxy phenyl phosphate hydrolyzed/min/25 C.

A test system consisting of Diazinon, 10 ppm, and 2 ml of buffer, and a buffer blank was prepared.

The enzyme (0.1 mg/ml H_2O) was added at zero time, and absorbancy recorded continuously at 245 nm for 26 min.

d. Phospholipase C:

Phospholipase C, or lecithinase C from Cl. welchi, catalyzes the hydrolysis between glycerol and phosphate (18).

$$\text{Lecithin} + H_2O \rightarrow \text{Diglyceride} + \text{Phosphorylcholine}$$

The enzyme preparation, with a specific activity of 1.6 units/mg, was diluted in water to contain 1 mg/ml, and 0.1 ml of this dilution was added to a tube containing 0.4 ml Tris buffer at pH 7.1 and 1 ml of stock ring-labeled Diazinon approximately equivalent to 13,000 cpm. Samples and controls

containing no enzyme were overlaid with 0.5 ml of ether, and incubated 24 hr at 37 C on a shaker. After incubation, the ether layer was evaporated under an air stream, and the culture extracted with 3 ml of benzene 3 times to remove the parent Diazinon. This extract was evaporated to reduce the volume, and placed on a planchette. The Diazinon concentration in the extract was determined as described in a gas flow counter.

RESULTS

I. Diazinon as a Nutritional Source:

A. Anaerobic Enrichment Cultures:

No growth occurred in any tube with Diazinon as the sole carbon source in anaerobic enrichment cultures (Table 4). After 7 days, light growth appeared in enrichments containing glucose and Diazinon, and after 24 hours, heavy growth in tubes containing yeast extract, casamino acids, and Diazinon.

Greater turbidity appeared in glucose control cultures than in glucose-Diazinon cultures, indicating either an inhibitory or selective action induced by Diazinon. This effect was not as apparent in cultures enriched with yeast extract and casamino acids.

B. Classification of Isolates:

Organisms isolated in enrichment cultures are classified by their gram stain reaction and morphology in Table 5. An array of 36 aerobes, and 18 anaerobes was used in this investigation. A short rod-shaped organism, D_{83}, previously implicated in Diazinon degradation (16), and a rod, designated AC, were further characterized for identification. D_{83}, the predominant organism isolated from Diazonon-treated soil, and AC, isolated from a saturated Diazinon solution, were classified as previously

TABLE 4.--Anaerobic enrichment cultures for the isolation of Diazinon-tolerant or utilizing populations

Enrichment	1		2		3		1		2		3		1		2		3	
Sample	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Carbon Source:																		
Diazinon	*		*		*		*		*		*		*		*		*	
Glucose							*	*	*	*	*	*						
Yeast Extract and Casamino Acids													*	*	*	*	*	*
No Carbon Source		*		*		*												
Growth	--	--	--	--	--	--	±	++	±	++	+	++	+++	+++	+++	+++	+	++

KEY:

Enrichment 1, Soil; 2, Soil; 3, Mud.

Sample A, Culture with Diazinon; B, Culture without Diazinon.

Growth, none - , very light ±, light +, moderate ++, heavy +++.

TABLE 5.--Description of isolates used in Diazinon studies

Aerobes	Anaerobes
Rods	
3A, 7C^{+}, 15A, 31^{+}, 42	3, 4, 5
51^{+}, 55, 57, 59^{+}, 78	7, 16,
86, 87, 88, 89, 90	7A, 7B
91, 92, 93, 94, 95	9P
96, 97, 98, 99, 100	
Acetobacter sp.	
Arthrobacter sp.	
Bacillus subtilis^{+}	
Mycobacterium phlei	
M. smegmatis	
Pseudomonas fluorescens	
Ps. sp. AC	
Coccobacilli	
1A	9, 9B, 13, 14
Diplobacilli	
	1, 6, 12
Cocci	
17	
Streptococci	
27^{+}, 31^{+}	2, 8, 15
Vibrio	10, 11
Filamentous Streptomyces	

KEY
g^{+} Gram positive

unrecorded Arthrobacter and Pseudomonas sp. (Table 6).

C. Replacement Cultures with Diazinon as the Sole Carbon, Sulfur, Phosphorus, and Nitrogen Source:

Since Pseudomonas AC was suspected of utilizing Diazinon as a nutritional source, it was inoculated into culture media with various elements of the original medium substituted with appropriate concentrations of Diazinon. In initial experiments all cultures increased in turbidity with time. Microscopic examination of the turbid cultures, however, revealed few cells, indicating little or no increase in cell number. Numerous refractile globules of Diazinon generated an increasing turbidity on shaking which mimicked growth. When this interference was eliminated, no turbidity appeared in cultures with Diazinon as the sole carbon, nitrogen, or phosphorus source, even after Diazinon had been solubilized in ethanol. No growth appeared in cultures with ethanol as the sole carbon source; however, control cultures supplemented with casamino acids did grow. Growth also appeared in flasks with Diazinon as the sole sulfur source, but did not recur on transfer.

D. Screening of Cultures with Diazinon as the Sole Carbon or Sulfur Source:

Since the ethanol and sulfur moieties were the most labile part of the Diazinon molecule, all aerobes, and anaerobes were screened for their ability to use either of

TABLE 6.--Characterization of organisms AC and D_{83}

	AC	D_{83}
Gelatin liquification	+after one week	+ after two weeks
Litmus milk	Clear red supernate White sediment	No change after seven days
Triple sugar iron agar	Slant: Pink Butt: Red No Gas	Slant: Yellow (acid) Butt: Yellow (acid) No Gas
Eosin Methylene Blue	Pink colonies	Pink colonies Since D_{83} was tentatively identified as an Arthrobacter sp., Arthrobacter globiformis and Arthrobacter citreus were grown as control cultures on EMB. Both produced pink colonies.
Nutrient agar	Smooth, entire pink colonies	Smooth, entire tan colonies
Fluorescence	++	+
Catalase	-	+
Growth Aerobic: nutrient agar slant Anaerobic: nutrient agar stab	 + -	 + -
Nutritional Requirements:	Prototroph stimulated by alanine	Prototroph stimulated by pooled amino acids or yeast extract
Generation time: Temperature Defined medium Nutrient broth	 37 C 120 hours 2.7 hours	 25 C 8.2 hours 2.5 hours

TABLE 6.--Continued

	AC	D_{83}
Temperature optimum	37 C	25 C
Spores	-	-
Gram reaction	Negative	Negative
India ink stain for capsules	-	-
Slime	-	Old cultures formed slime and produced bizarre forms
Motility: wet mount	+	+
Electron micrographs Negative stain		
Flagella	Lophotrichous (6-8 flagella)	Monotrichous (A few cells had a degenerate sub-polar flagellum)
Size		
Length	3.5 u	2.7 u
Width	1.0 u	0.83 u

the 2 elements. No growth occurred after 5 days in any culture with Diazinon as the sole carbon source. Seven cultures grew with Diazinon as the sole sulfur source (Table 7). When these 7 were transferred to MM with a Diazinon sulfur source, with the exception of 84 and 42, they failed to grow after 2 transfers. These 2 cultures grew through 5 transfers; however, cells in the Diazinon-treated cultures appeared under stress. They contained numerous refractile inclusions absent in the control cultures.

E. Location of Diazinon in Cultures by the Ammonium Palladium Chloride Test for P=S:

Cultures which grew with a Diazinon sulfur source on the initial transfer had traces of Diazinon in the benzene extract of the supernate, and a significantly increased amount in the extract of supernate and cells (Table 8). This result indicated adherence of Diazinon on the cells, or incorporation into the cells. The adherence of Diazinon appeared to be associated with lysing in treated cells.

II. Effects of Diazinon or Breakdown Products on Bacteria:

A. Effects of Diazinon on Generation Time:

The generation times of the 17 soil isolates were increased when grown anaerobically in cultures supplemented with Diazinon (Table 9). The effect was most apparent after 2 hours. The yield of cells was correspondingly lower in

TABLE 7.--Growth of aerobic bacteria with Diazinon as the sole carbon or sulfur source

Culture No.	Control	Diazinon Carbon Source	Diazinon Sulfur Source
59	+	-	-
31	+	+ Possible	-
27	+	-	+
Streptomyces sp.	+	-	-
83	+	-	-
84	+	-	++
55	+	-	-
Mycobacterium smegmatis	+	-	-
57	+	-	-
17	-	-	++
Bacillus cereus	+	-	-
42	+	-	++
3	+	-	++
Bacillus subtilis	+	-	-
15	+	-	-
1	+	-	+
78	+	-	-
51	-	-	-
7	+	-	++

TABLE 8.--Presence of Diazinon in cultures grown with Diazinon as a sole sulfur source

	Supernate		Supernate and Cells	
Culture	Rf	Intensity	Intensity	Rf
7	-	-	+++	0.74
83	-	-	±	0.77
84	0.76	+	±	0.74
Control	0.67	++	+++	0.74
3	0.7	±	++	0.69
42	0.7	±	+	0.69
31	0.66	±	+	0.66
Control	0.68	+++	+++	0.68
1	0.66	±	+	0.67
17	0.66	±	++	0.71
Control	0.68	+++	+++	0.75

KEY:

Detection of Diazinon by the ammonium palladium chloride test for P=S

Intensity of Yellow Color
None - Light ++
Barely Visible ± Dark +++
Very Light +

TABLE 9.--The influence of Diazinon on the generation times of selected anaerobic cultures at the third hour of growth

Culture	Generation Times		pH	
	Control	Diazinon	Control	Diazinon
1	1.20	3.50	5.65	5.40
2_A	1.25	3.00	5.10	4.30
3	0.85	2.85	5.90	5.30
4	1.05	2.80	5.70	5.10
5	1.15	1.95	5.80	5.39
6	1.15	3.70	5.65	5.35
7	0.70	3.70	5.55	5.35
8	1.00	5.00	5.40	5.38
9	1.00	6.60	6.01	6.80
10	1.90	5.50	6.55	6.20
11	1.00	10.50	5.50	5.35
12	1.30	12.80	5.15	5.30
13	1.10	12.70	5.80	5.35
14	1.20	4.10	5.60	5.85
15	0.85	4.00	5.50	5.75
16	1.20	0.00	5.60	5.55
17	1.10	1.90	5.70	7.00

treated cultures (Figs. 8, 10, 12). Aerobes similarly treated also had increased generation times (Table 10). Usually, when aerobes, or anaerobes were grown in an enriched medium, little or no change was manifested in the growth rate. This was in agreement with previous observations with such cultures supplemented with yeast extract, and casamino acids where it was noted that cultures were very turbid (Table 4).

B. Effects of 2-Isopropyl-4-Methyl-6-Hydroxypyrimidine, the Ammonium Salt of Diethyl Thiophosphoric Acid, Ethanol, or Diazinon on Generation Times of Anaerobically Growing Bacteria:

Since anaerobes were known to cause the hydrolysis of the parent molecule to ring, side chain and ethanol, these moieties were tested for their effects on growth rates (Table 11). Diazinon caused an increased generation time in cultures 9P, 7, 6, and 9 of 56%, 28%, 25%, and 15%, respectively. Cultures 6 and 7 autolyzed faster in the Diazinon-treated cultures than in the control, side chain, ring, or ethanol treated cultures. Controls of 6 and 7 autolyzed after 24 hours, establishing the fragility of the cells.

The 2-isopropyl-4-methyl-6-hydroxypyrimidine treated cultures responded similarly to the controls with regard to growth rates and final cell yields (Table 11). The ammonium

Fig. 7.--Growth curves of anaerobes 1-5.
Controls: No Diazinon present.

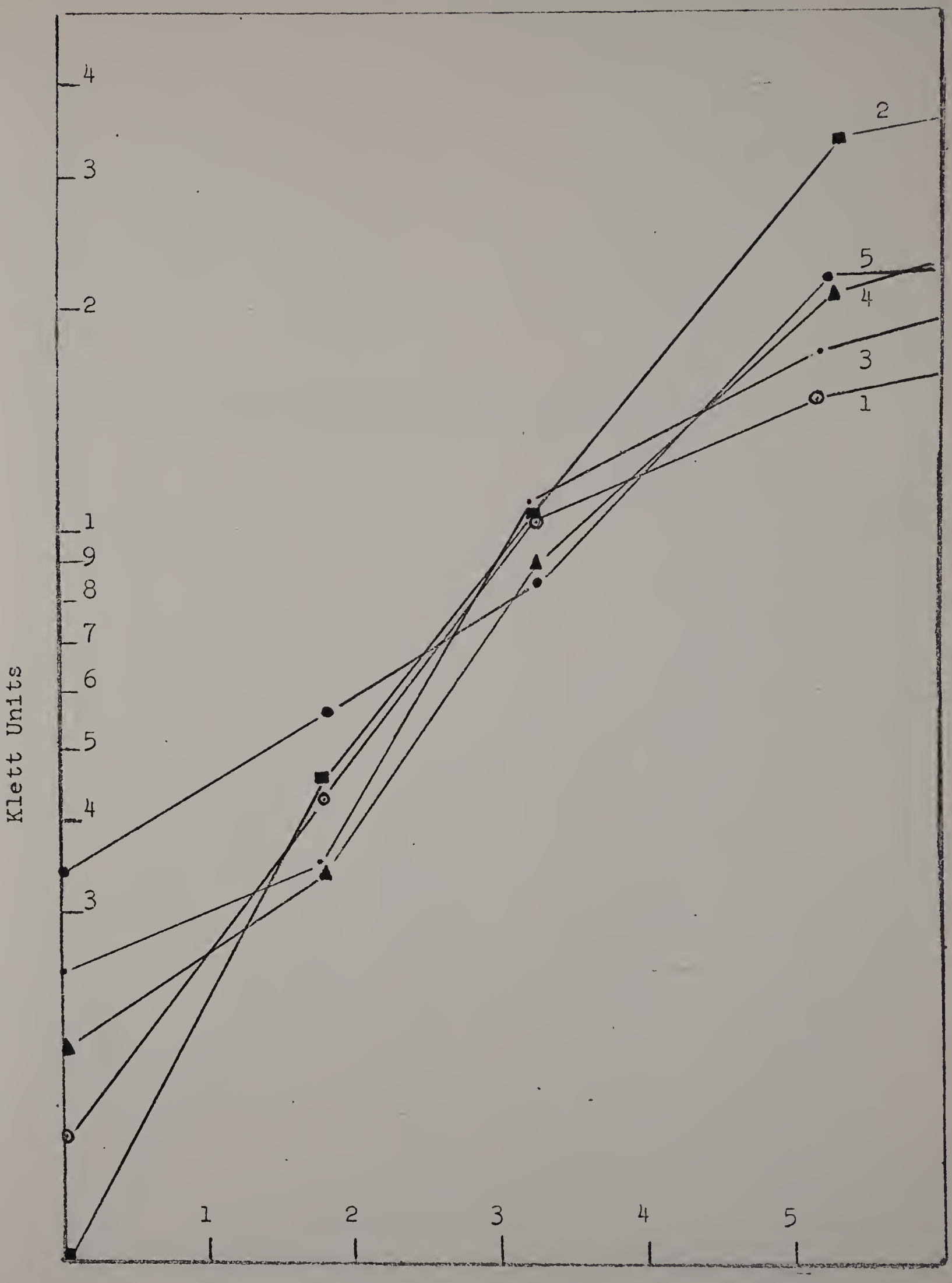

Klett Units
4
3
2
1
9
8
7
6
5
4
3
2
5
4
3
1
1
2
3
4
5
Time (hr)

Fig. 8.--Growth curves of anaerobes 1-5 in medium supplemented with Diazinon.

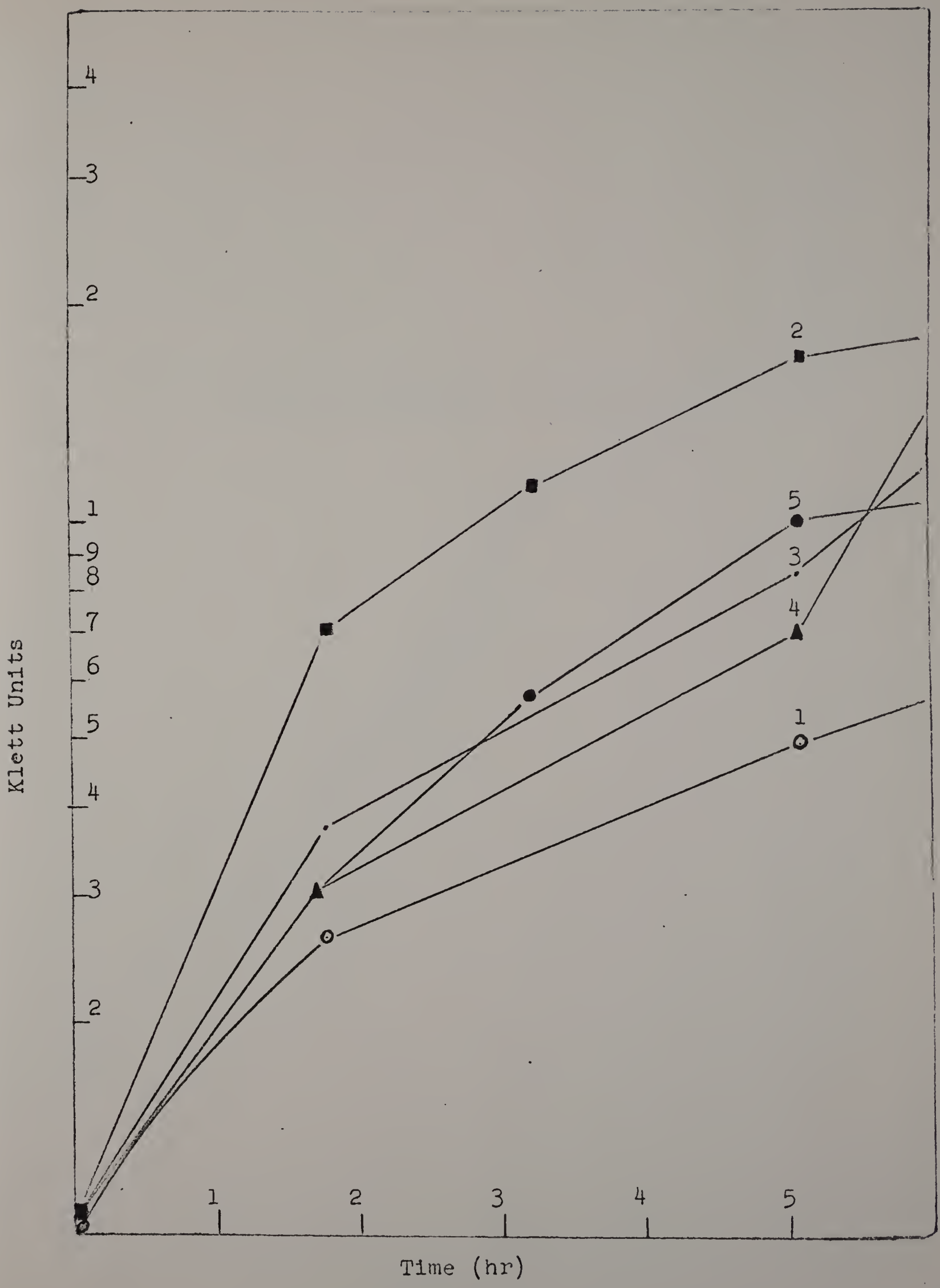

Klett Units
4
3
2
1
9
8
7
6
5
4
3
2
1
2
3
4
5
1
2
3
4
5
Time (hr)

Fig. 9.--Growth curves of anaerobes 6-10.
Controls: No Diazinon present.

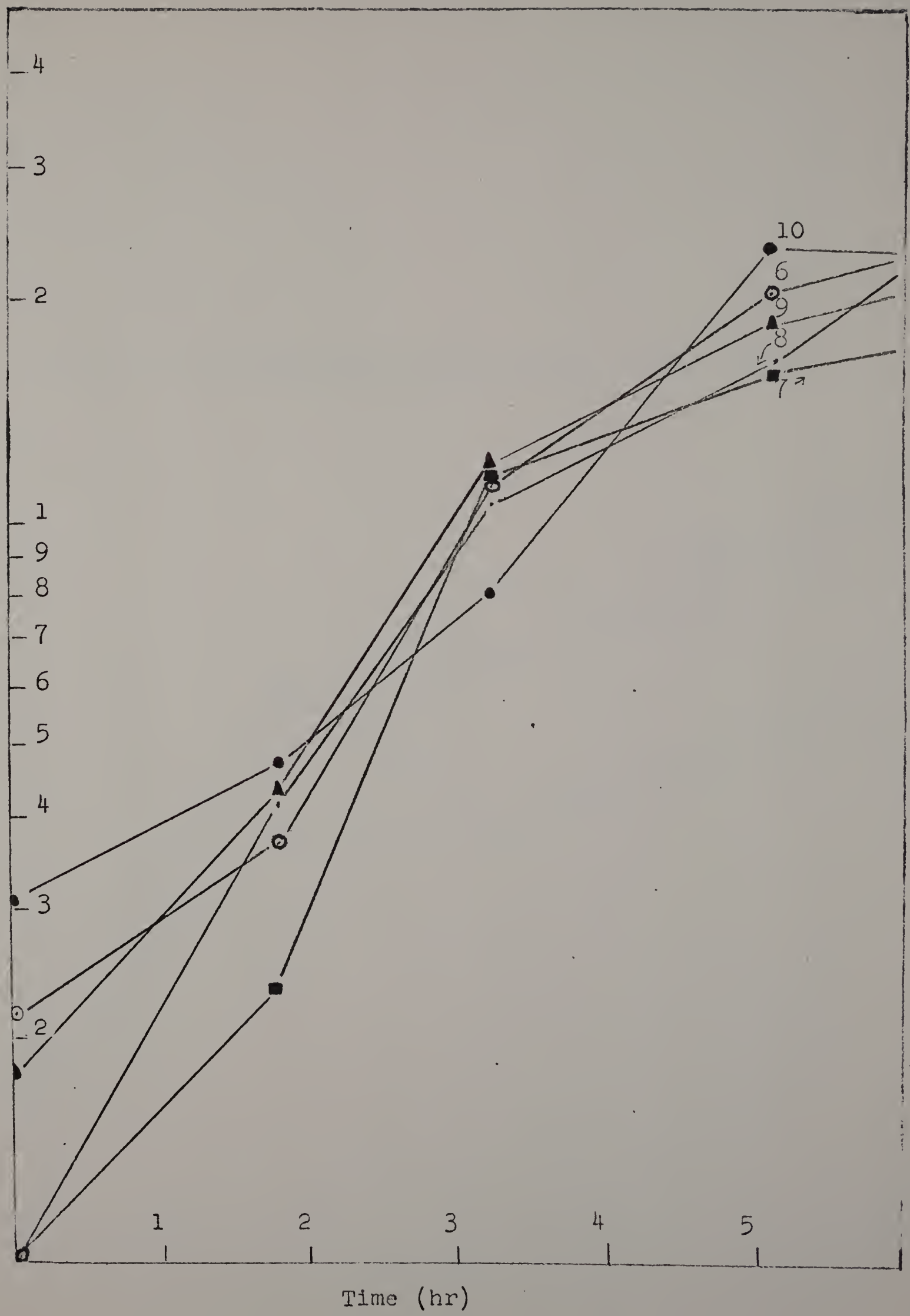

Klett Units
4
3
2
1
9
8
7
6
5
4
3
2
1
10
6
9
8
7
1
2
3
4
5
Time (hr)

Fig. 10.--Growth curves of anaerobes 6-10 in medium supplemented with Diazinon.

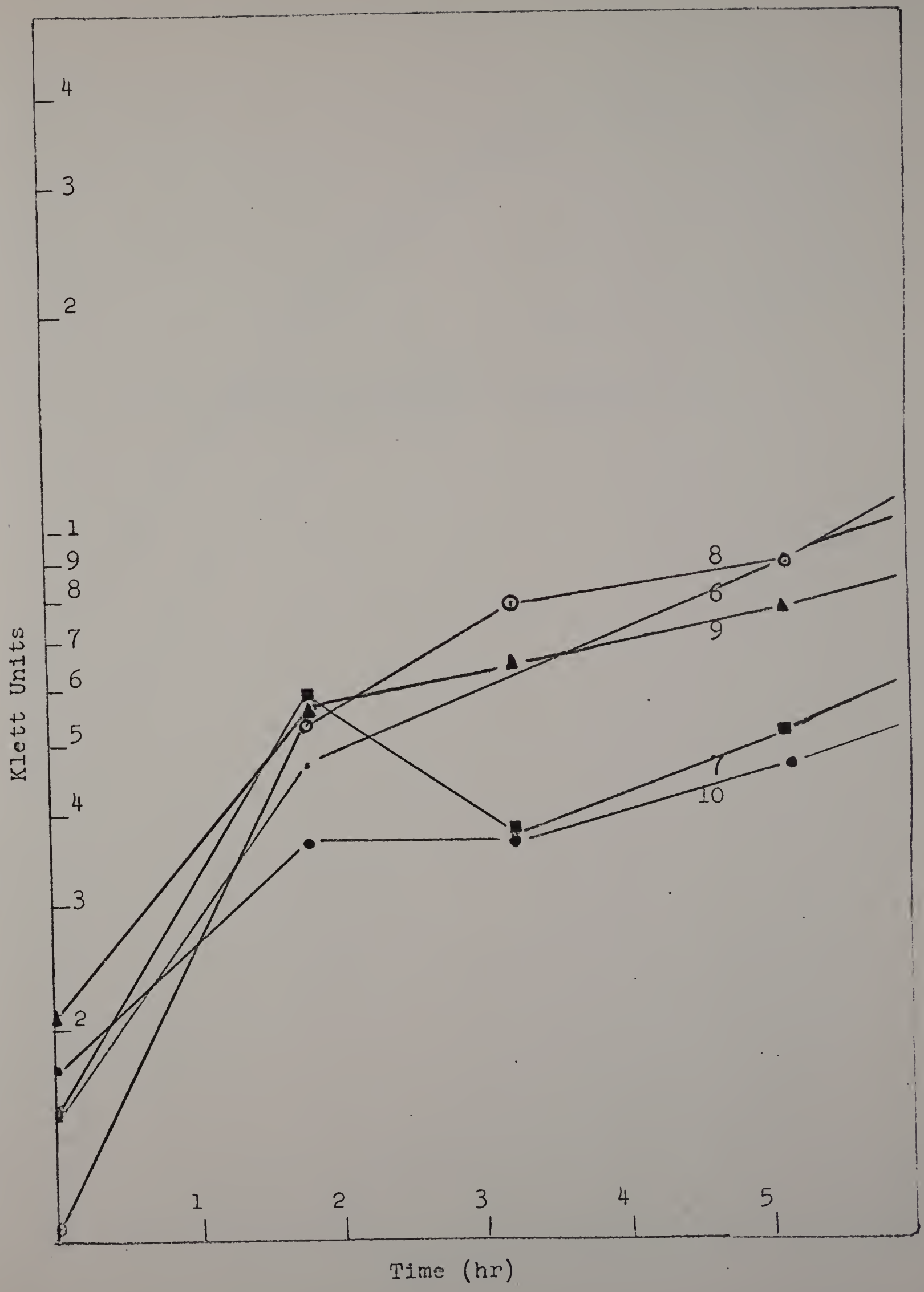

Klett Units
4
3
2
1
9
8
7
6
5
4
3
2
1
2
3
4
5
Time (hr)
8
6
9
7
10

Fig. 11.--Growth curves of anaerobes 11-13.
Controls: No Diazinon present.

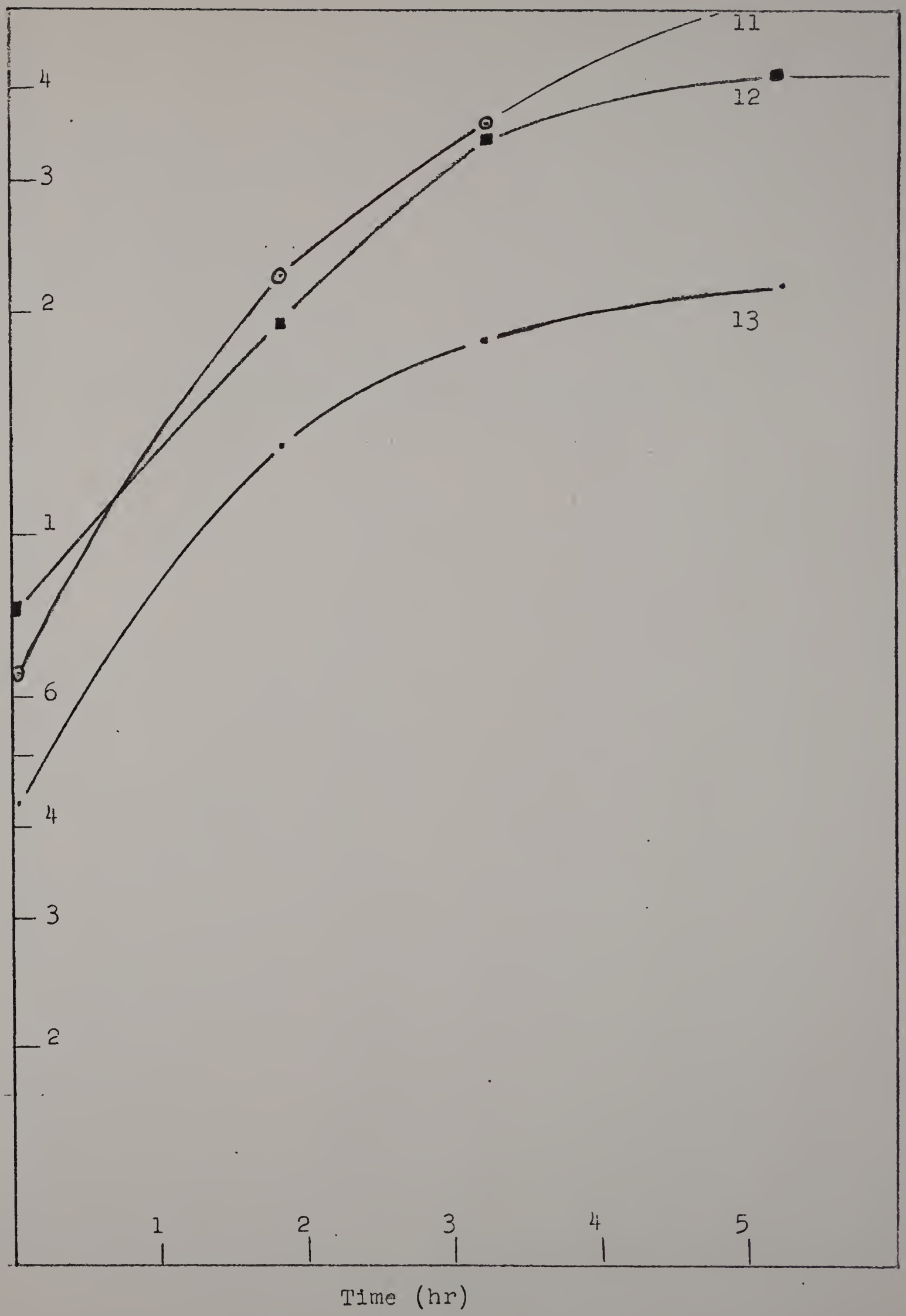

11
12
13
4
3
2
1
6
4
3
2
Klett Units
1
2
3
4
5
Time (hr)

Fig. 12.--Growth curves of anaerobes 11-13 in medium supplemented with Diazinon.

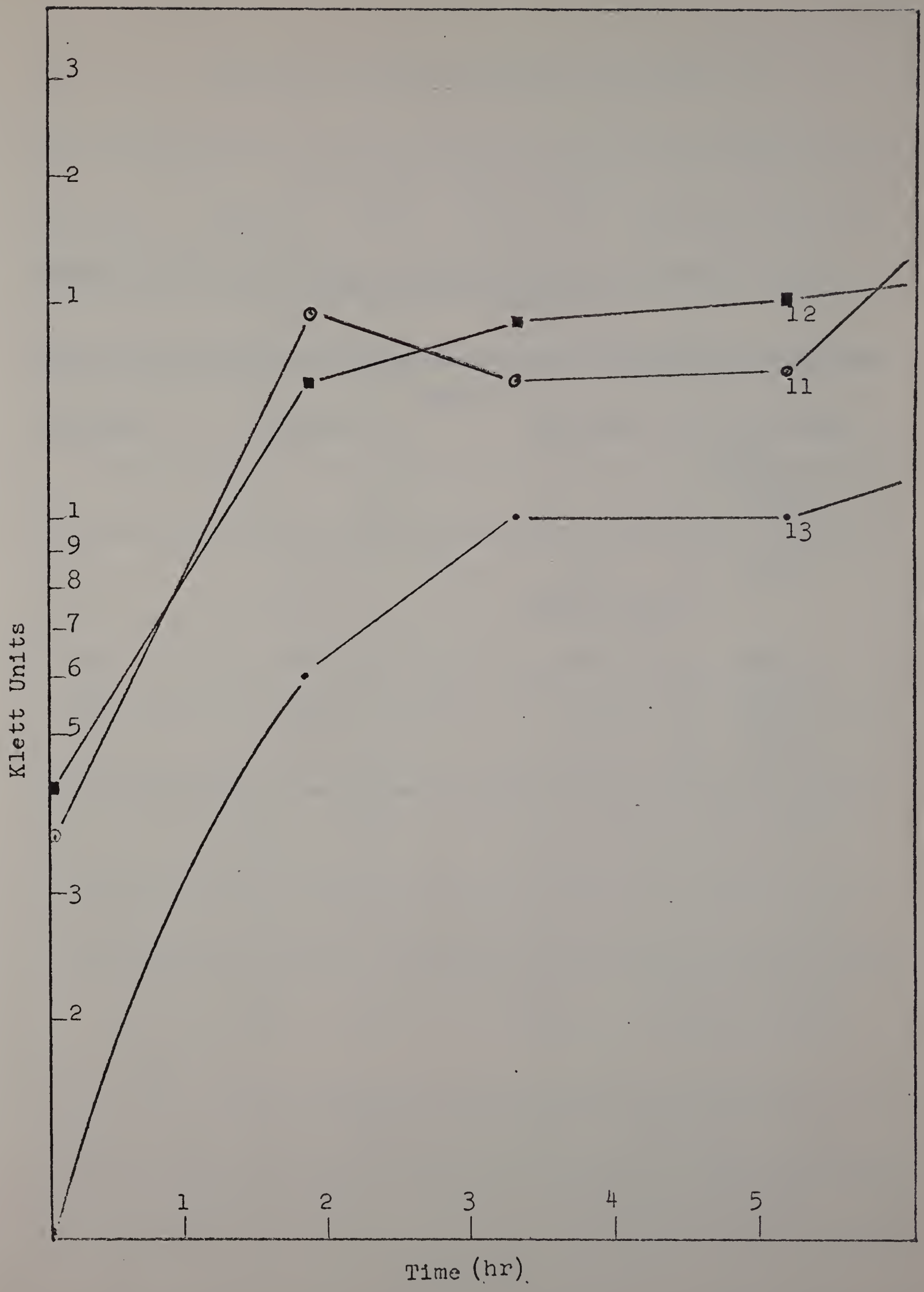

3
2
1
1
9
8
7
6
5
3
2
Klett Units
12
11
13
1
2
3
4
5
Time (hr)

TABLE 10.--The influence of Diazinon on generation times of selected aerobes

Culture	Hours		
	Control	Diazinon	% Increase in Generation Times
1	3.3	4.7	42%
17	4.1	Inhibition	-
84	8.3	11.2	35%
42	2.6	3.9	50%
7	6.1	7.0	15%

TABLE 11.--Generation times of anaerobes treated with Diazinon, or its breakdown products

Culture	Generation Time (Hours) Control	Ring	Side Chain	Ethanol	Diazinon
9 Proteus	1.25	1.2	1.8	1.9	1.6
	1.35	-	1.8	1.6	-
	2.1	2.1	3.1	3.0	3.2
Av.	1.53	1.6	2.2	2.2	2.4
7	2.7	3.9	-	7.1	4.3
	3.0	3.2	3.3	6.1	3.1
	3.0	-	3.4	5.5	-
Av.	2.9	3.5	3.4	6.2	3.7
6	2.1	2.2	2.5	2.2	2.7
	2.5	2.5	4.0	3.7	3.2
	2.7	-	3.5	4.5	-
Av.	2.4	2.4	3.3	3.5	3.0
9	1.1	1.3	1.7	1.8	1.6
	1.5	1.5	1.4	1.34	1.4
Av.	1.3	1.4	1.6	1.6	1.5

salt of diethyl thiophosphoric acid and ethanol produced increased generation times (Table 11). Since side chain and ethanol seemed to have similar effects on generation times, an experiment was conducted to see if generation times would vary directly with concentration.

In most cultures, equivalent concentrations of side chain and ethanol produced similar increases in generation times, and growth rate was directly related to concentration of the compounds (Table 12). In culture 7, however, ethanol had a more pronounced effect than side chain (Table 12).

These results indicated that Diazinon, side chain and ethanol, but not the pyrimidine 1 ring compound, were the probable determinants in population selection.

In most cultures, longer generation times were correlated with decreased yields, except in culture 9B, a Pseudomonas sp. In this case, even though Diazinon, side chain and ethanol treated cultures had longer generation times when compared to control or ring treated cultures, the Diazinon, side chain and ethanol-treated cultures had higher yields of cells, and shorter lag periods (Table 13).

C. Identification of 2-Isopropyl-4-Methyl-6-Hydroxy-pyrimidine:

IR spectra showed no absorption in the hydroxy region, 3,500-3,600 cm^{-1}, and strong absorption in the carbony 1 region, 16,500-17,000 cm, indicating that the

TABLE 12.--Comparison of effects of equivalent amounts of ethanol and Diazinon side chain on generation times of anaerobes 6 and 7

	Generation Time (Hr)			
Organism	6		7	
Diazinon Equivalent ppm	EtOH	Side Chain	EtOH	Side Chain
40	2.2	2.5	--	--
102	2.95	2.95	3.3	3.1
1,502	3.5	3.4	5.1	3.4
31,000	4.5	4.0	6.1	4.0
Control	2.5	2.5	3.0	3.0

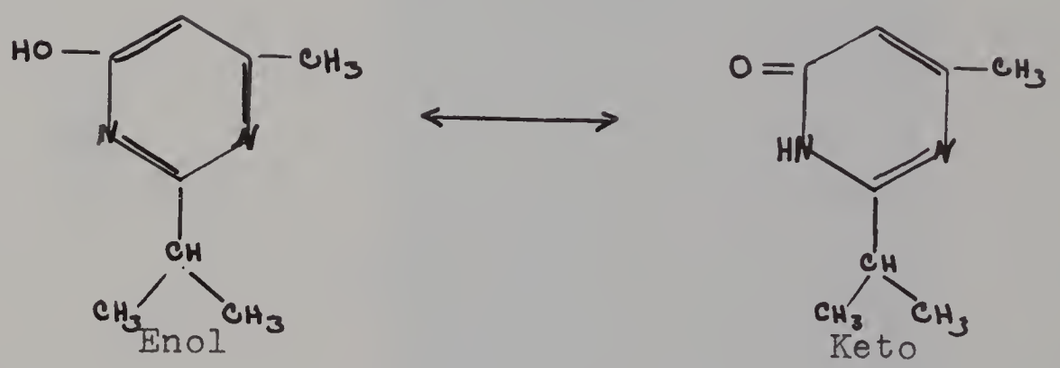
TABLE 13.--Comparison of lag periods of culture 9B with various supplements

Hours				
Control	Ring	Side Chain	EtOH	Diazinon
3	3	1	1	1

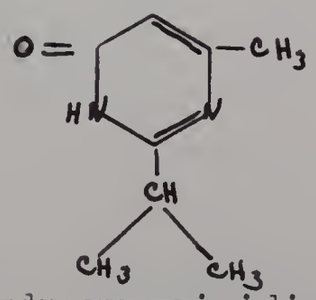
OH-pyrimidine existed almost exclusively in the keto form (Fig. 13).

D. Effects of Diazinon on Total Nucleic Acid Synthesis:

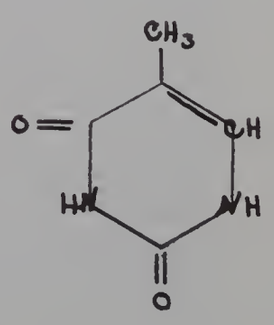
The solvent partition studies with C^{14} ring-labeled Diazinon showed that it hydrolyzed into 2-isopropyl-4-methyl-6-hydroxypyrimidine and diethyl thiophosphoric acid. The ring compound isomerized and reached equilibrium between the keto and the enol forms. Equilibrium strongly favored the keto form.



There was a striking similarity between the pyrimidine, thymine, and the keto form of the 2-isopropyl-4-methyl-6-hydroxypyrimidine.

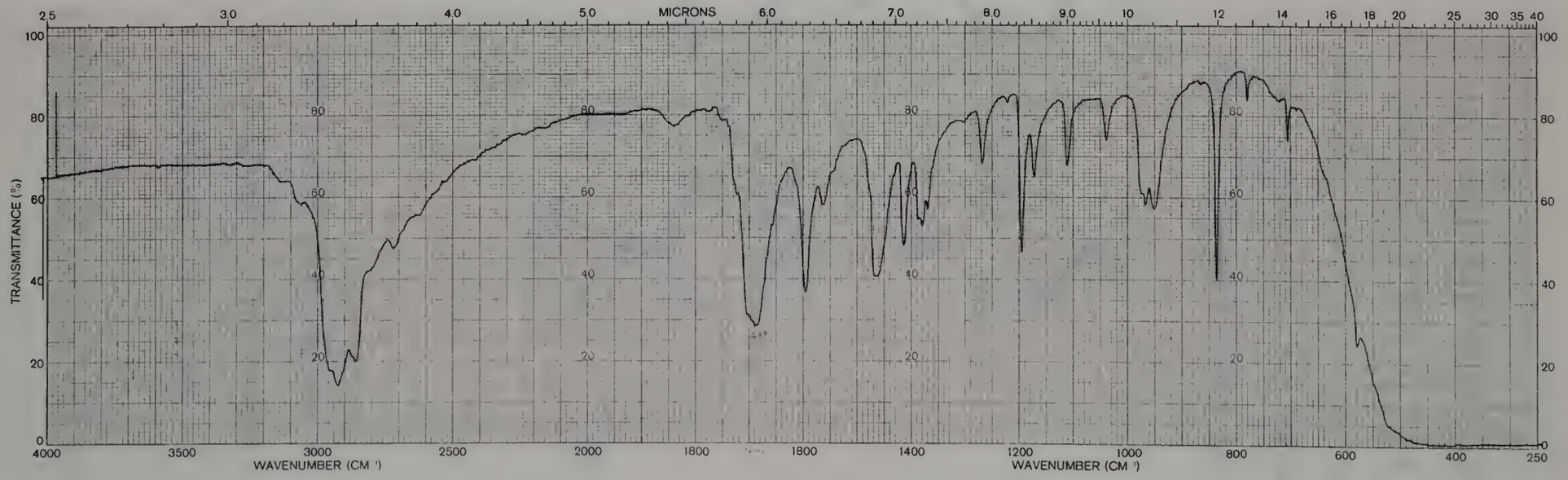


Hydroxypyrimidine

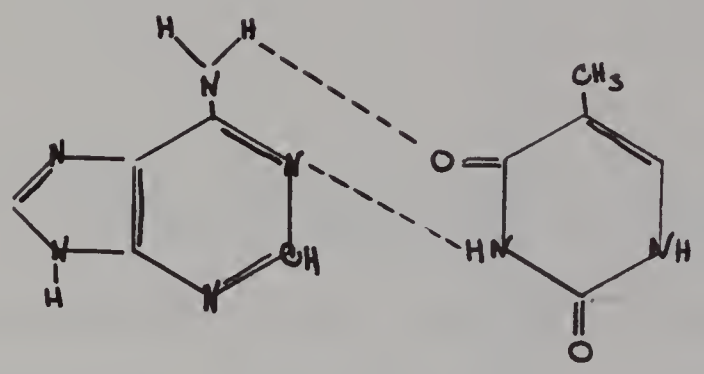


Thymine

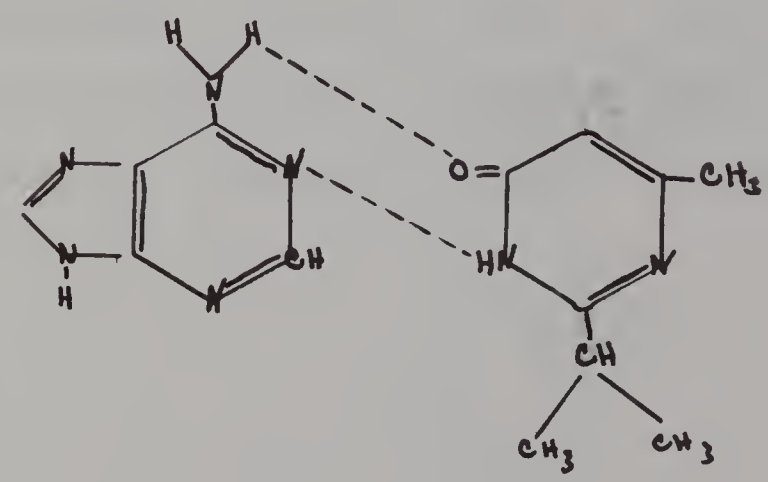
Fig. 13.--Infrared absorption spectra of 2-isopropyl-4-methyl-6-hydroxypyrimidine.


MICRONS
2.5
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10
12
14
16
18
20
25
30
35
40
TRANSMITTANCE (%)
100
80
60
40
20
0
4000
3500
3000
2500
2000
1800
1800
1400
1200
1000
800
600
400
250
WAVENUMBER (CM ⁻¹)
WAVENUMBER (CM ⁻¹)

A possibility arose that the ring compound was being incorporated into the DNA or RNA of the cell in amounts not detectable by our methods, causing a decrease in the rate of total nucleic acid synthesis, and hence, a decrease in the growth rates. This is shown in the diagram below.



Adenine Thymine



Adenine Hydroxypyrimidine

Total nucleic acids were determined in anaerobes 6, 7, 9B and 9P. In all cultures, the rate of synthesis in the Diazinon-treated cultures was the same as the controls (Figs. 14, 15).

E. Cytological Effects of Diazinon and Its Breakdown Products:

Changes, such as lysis, clumping, spheroplast

Fig. 14.--Effects of Diazinon on total nucleic acid synthesis of anaerobes 6 and 7

a. Culture 6 Control (no Diazinon) ■—■—■

b. Culture 6 Test (Diazinon) □—□—□

c. Culture 7 Control (no Diazinon) ●—●—●

d. Culture 7 Test (Diazinon) o—o—o

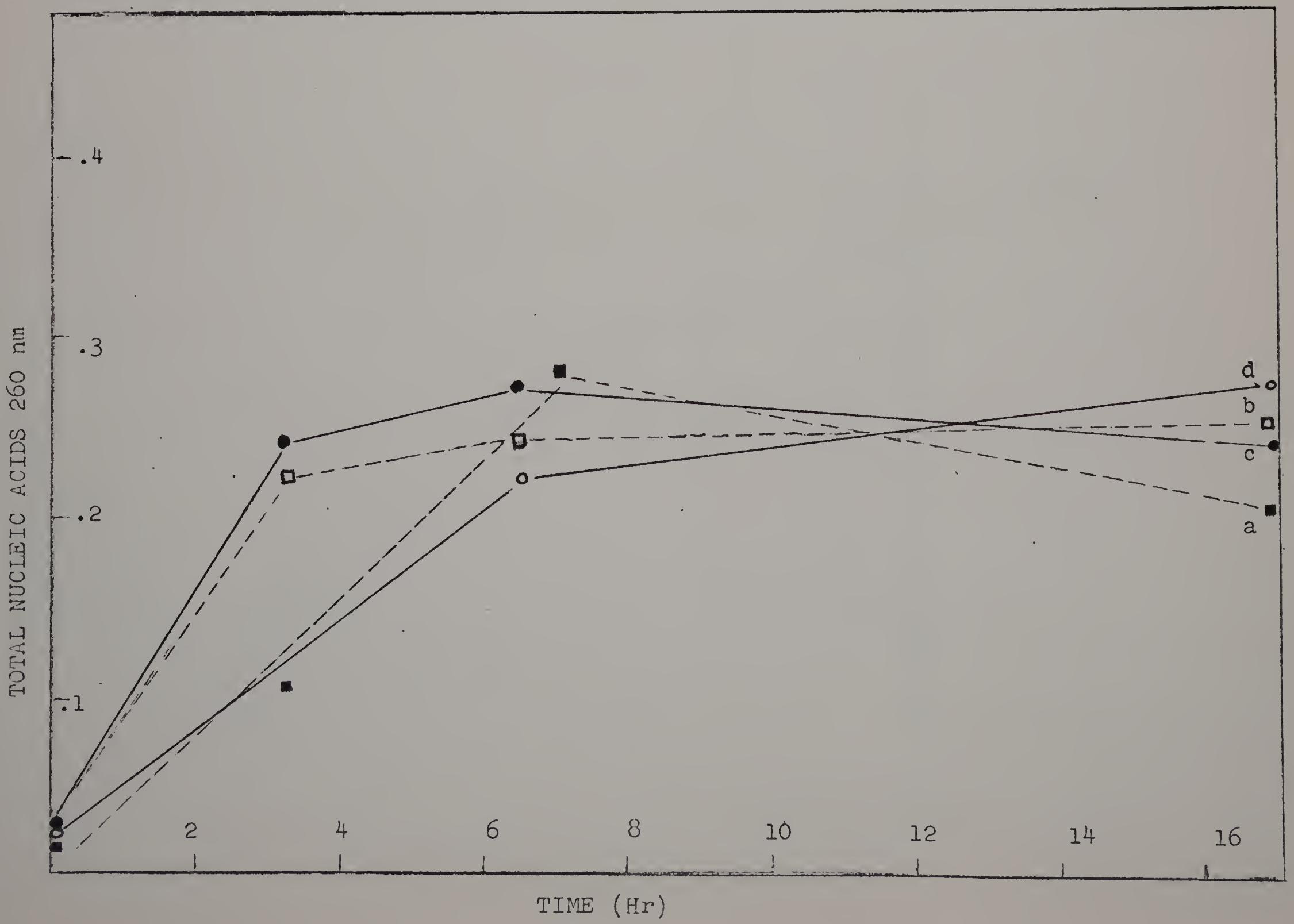

TOTAL NUCLEIC ACIDS 260 nm
.4
.3
.2
.1
2
4
6
8
10
12
14
16
TIME (Hr)
a
b
c
d

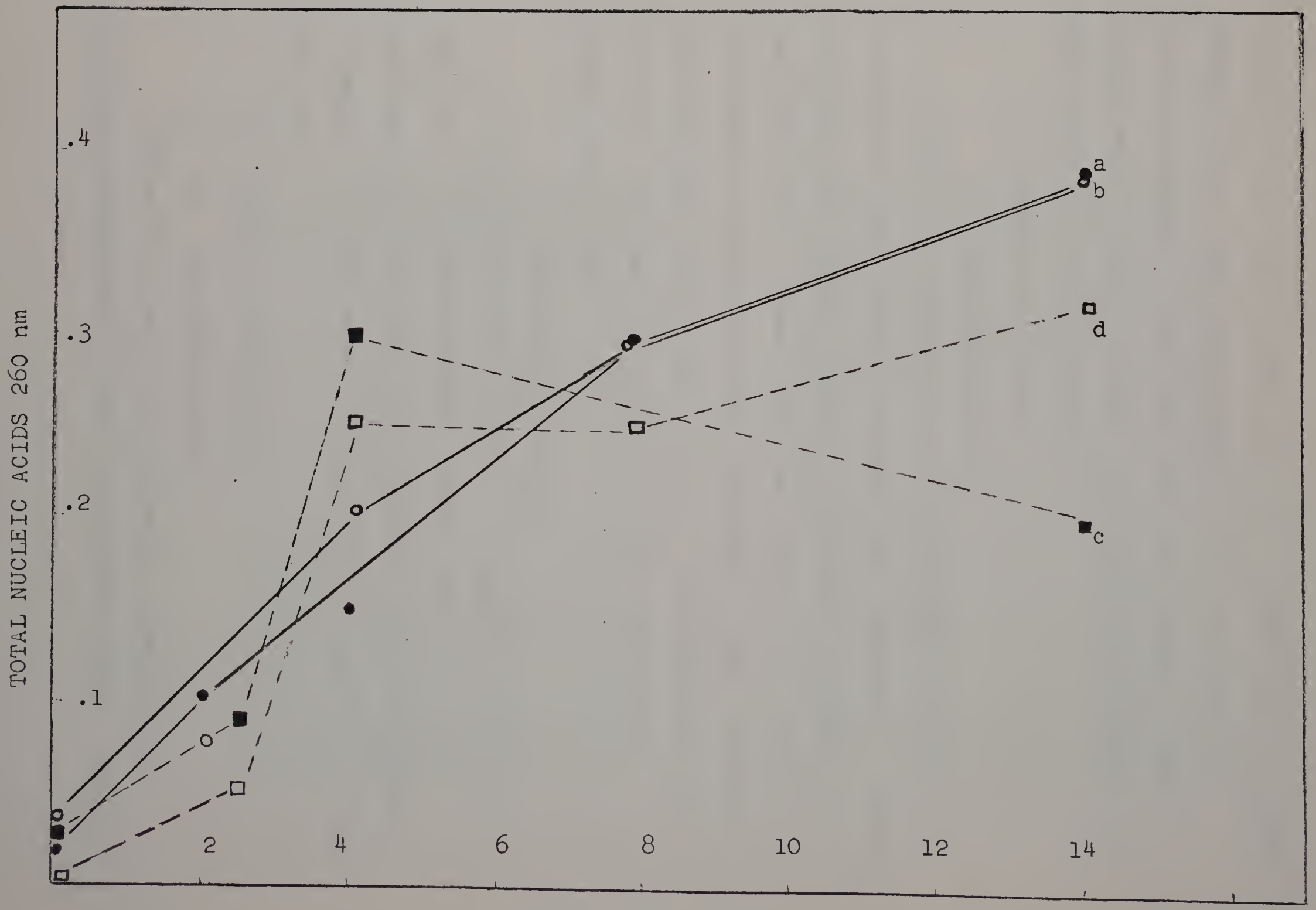
Fig. 15.--Effects of Diazinon on total nucleic acid synthesis of anaerobes 9B and 9P

a. Culture 9B Control (no Diazinon) ●—●—●

b. Culture 9B Test (Diazinon) o—o—o

c. Culture 9P Control (no Diazinon) ■—■—■

d. Culture 9P Test (Diazinon) ▫—▫—▫


TOTAL NUCLEIC ACIDS 260 nm
.4
.3
.2
.1
2
4
6
8
10
12
14
TIME (Hr)
a
b
c
d

formation, and loss of motility were visible in a wet mount preparation by phase microscopy of cells treated with Diazinon, ethanol, side chain and ring compound. These changes were recorded by phase and electron micrographs of whole cells and of thin sections.

1. Effect on Culture 6:

a. Phase Microscopy:

Lysis was present in Diazinon-treated cells as evidenced by much debris and spheroplast formation. Ethanol and side-chain treated cultures contained far fewer cells than the control. These cells were shorter than normal. Ring-treated cultures and Diaz treated cultures contained long and normal cells (Fig. 16 a, b). The Diazinon culture also contained cells which divided, but did not separate (Fig. 16b).

b. Electron Microscopy:

Spheroplasts were visible in negative stain preparations of Diazinon-treated cultures (Fig. 17 a, b). The fragility of the cells was evident as the preparation of samples for thin section caused loss of rigidity of the cell walls.

2. Effect on Culture 7:

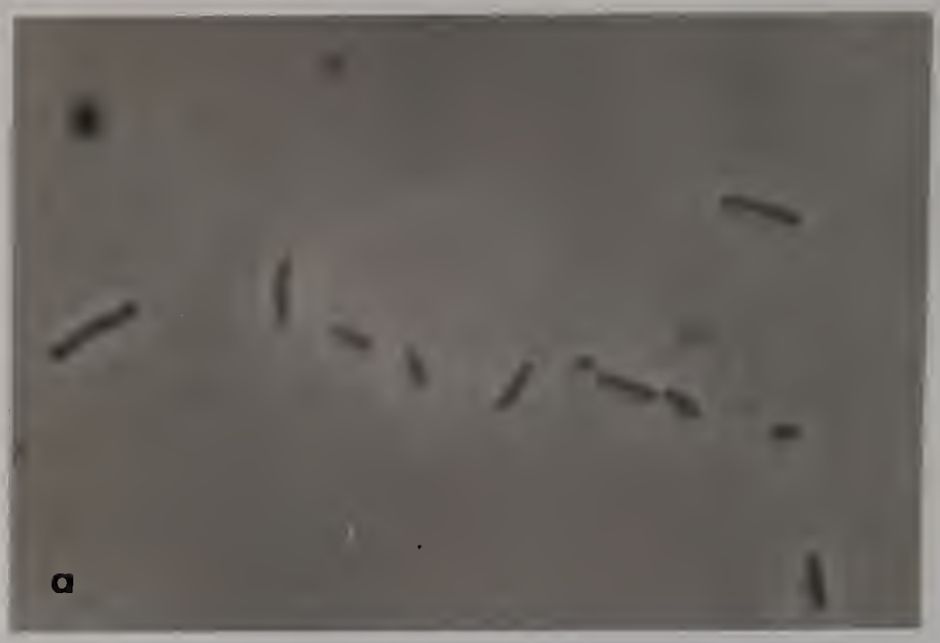
a. Phase Microscopy:

Cells of culture 7 occasionally formed regular pallisade arrangements in early stationary phase. All of

Fig. 16.--Phase contrast micrographs of culture 6

a. Control

b. Diazinon-treated culture. Long cells, division, but no separation (pinching).


a

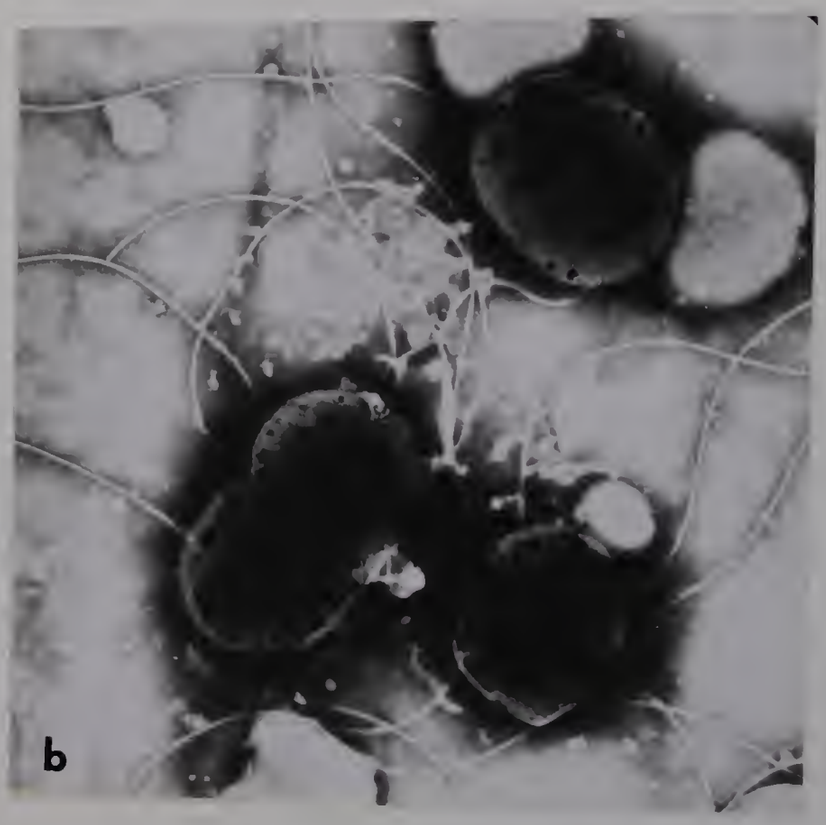

b

Fig. 17.--Electron micrographs of negative stains of culture 6

a. Control

b. Diazinon-treated culture. Spheroplast formation.


a


b

the following pictures were taken when cultures were in log phase. There was no clumping or pallisade formation in the control (Fig. 18a).

In Diazinon-treated cultures, cells frequently clumped in groups of 3 to 5 cells or more (Fig. 18b). Many cells appeared translucent under phase contrast, indicating cell death. There were significantly more degenerate, lysing cells, and spheroplast formation in the treated culture than in the control. Numerous cells also adhered to the Diazinon globules, surrounding them completely.

Cultures treated with ethanol or side-chain contained far fewer cells than the control, though these cells were normal in appearance. Ring-treated cultures were similar to the controls both in number and appearance.

b. Electron Microscopy:

Negative stains revealed clumping and spheroplast formation (Fig. 19 a, b), in the presence of Diazinon. Because of their fragility, when cells of culture 7 were prepared for thin sectioning, the cell wall collapsed, as in culture 6, so that definitive discrepancies between treated and untreated cells could not be assessed.

3. Effect on Culture 9B:

a. Phase Microscopy:

Culture 9B appeared normal in phase micrographs of ring, side-chain, ethanol and Diazinon-treated cultures;

Fig. 18.--Phase contrast micrographs of culture 7

a. Control

b. Diazinon-treated culture. Clumping ghost cells, lysing.


a


b

Fig. 19.--Electron micrographs of negative stains of culture 7

a. Control

b. Diazinon-treated culture. Clumping, spheroplast formation.


a


b

however, the cells in the Diazinon-treated culture were less motile than the control. A flagellar stain preparation** showed significantly fewer flagella on cells in the treated culture.

b. Electron Microscopy:

Negative staining revealed fewer flagella on the cells of the Diazinon-treated culture than in the control (Fig. 20 a, b). This explained the weak motility of the wet mount preparation of the Diazinon culture.

4. Effect on Culture 9P:

a. Phase Microscopy:

All cultures exhibited a degree of lysis, and all were only weakly motile. When the Diazinon-treated culture (Fig. 21a) was retransferred a second time to a medium containing Diazinon (Fig. 21 c, d) and a control (Fig. 21b), the cultures manifested marked differences in 24 hours. There was profuse lysis in the Diazinon culture, and some cells divided abnormally, pinching in several places without dividing, or pinching at one end instead of the middle (Fig. 21 c, d). Many cells were translucent "ghost" cells.

5. Effect on Culture 11:

a. Phase Microscopy:

Cultures treated with Diazinon underwent marked

**Appendix II.

Fig. 20.--Electron micrographs of negative stains of culture 9B

a. Control

b. Diazinon-treated culture. Fewer flagella than control.


a

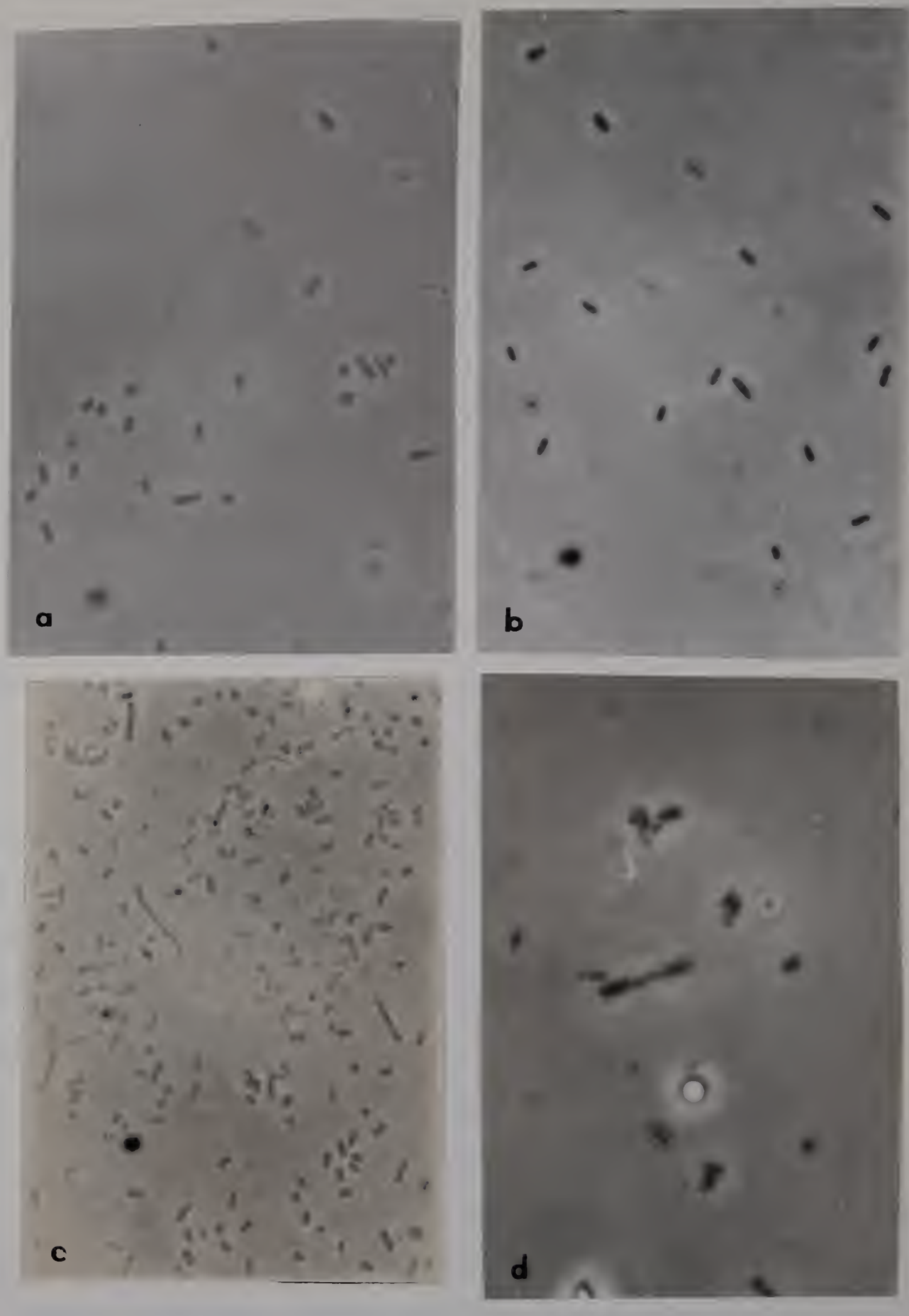

b

Fig. 21.--Phase contrast micrographs of culture 9P

a. Original Diazinon-treated culture

b. Control: Original Diazinon-treated culture after 24 hr growth in Diazinon-free medium

c., d. Diazinon-treated culture: Original Diazinon-treated culture after second transfer into Diazinon-containing medium. Pinching, ghost cells, long forms, lysing.


a
b
c
d

lysis, while the controls appeared normal (Fig. 22a, b).

b. Electron Microscopy: Neg. Staining

Most cells were lysed, and the few remaining cells were rarely in chains, and not as well formed into the vibrio shape of the control (Fig. 23 a, b).

6. Effect on Mycobacterium phlei:

a. Phase Microscopy:

Visible clumping of cells was apparent in Diazinon-treated cultures of the bacterium, M. phlei. Microscopic examination established that there was gross clumping of cells in tangled, fused masses (Fig. 24 b, c). Cells in the control were longer than in the treated sample, and these cells were not clumped (Fig. 24a).

b. Electron Microscopy:

Diazinon-treated cells also appeared clumped in micrographs of thin sections, and some portions of the cell walls seemed joined. Cells of the treated culture were coated with a black, amorphous film (Figs. 25, 26) and the membrane appeared thickened and in a possibly pre-lytic stage.

7. Effect on R. rubrum M_2B:

a. Electron Microscopy:

Negative stains of R. rubrum M_2B, treated with Diazinon, showed abnormal division, with cells pinching, as in culture 9P, but not dividing. There was also increased lysis in the

Fig. 22.--Phase contrast micrographs of culture 11

a. Control

b. Diazinon-treated culture. Almost complete lysis.


a

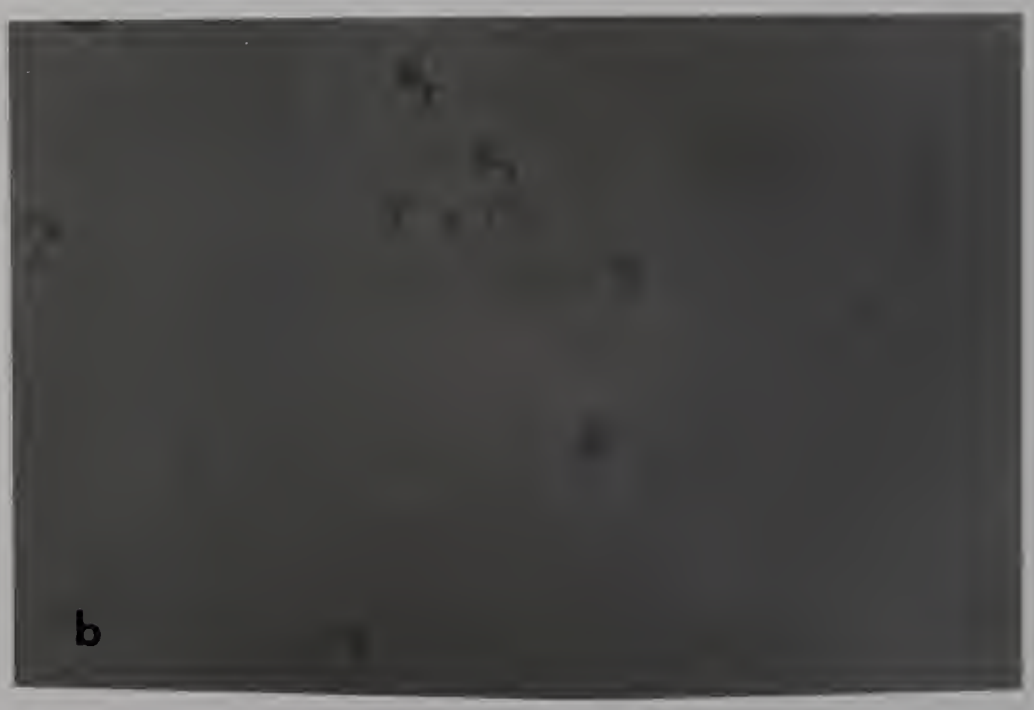

b

Fig. 23.--Electron micrographs of negative stains of culture 11

a. Control. Vibrio form in chains.

b. Diazinon-treated culture. Singly, less curved.

a

b

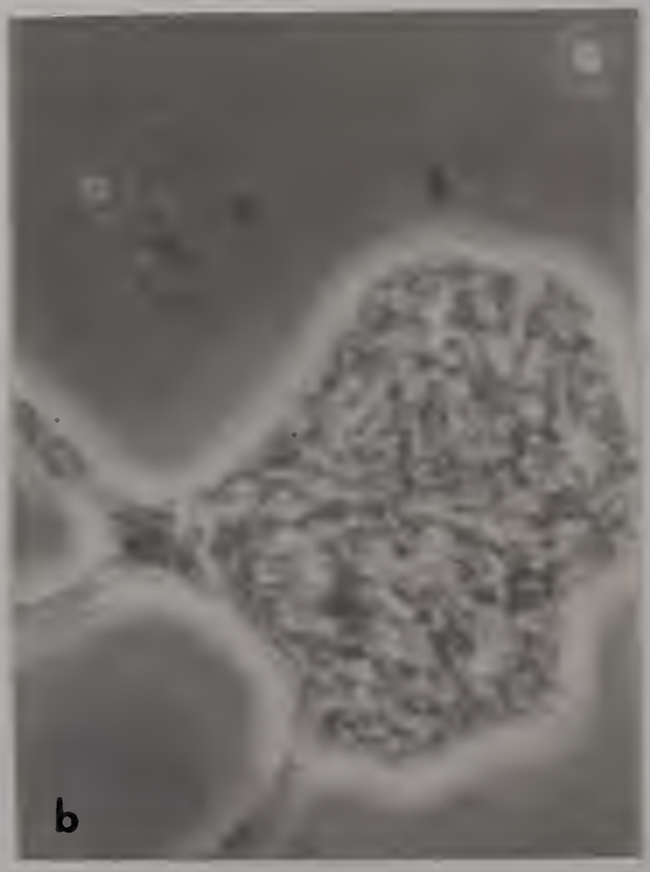
Fig. 24.--Phase contrast micrographs of Mycobacterium phlei

a. Control. Cells single, longer than in Diazinon cultures.

b. Diazinon-treated culture. Clumping in fused masses.

c. Diazinon-treated culture. Clumping in fused masses.


a


b


c

Fig. 25.--Electron micrographs of thin sections of *Mycobacterium phlei*

Control

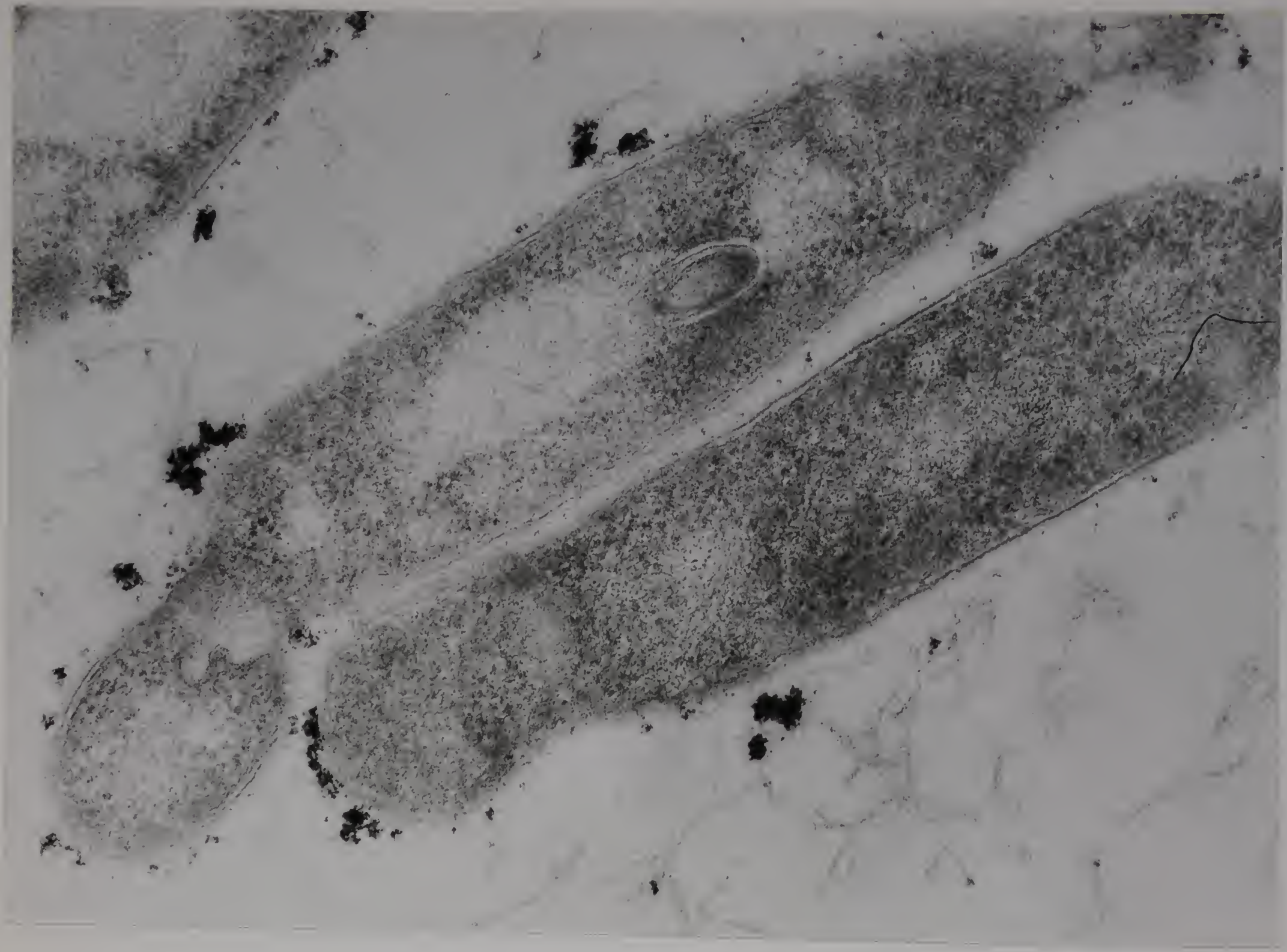
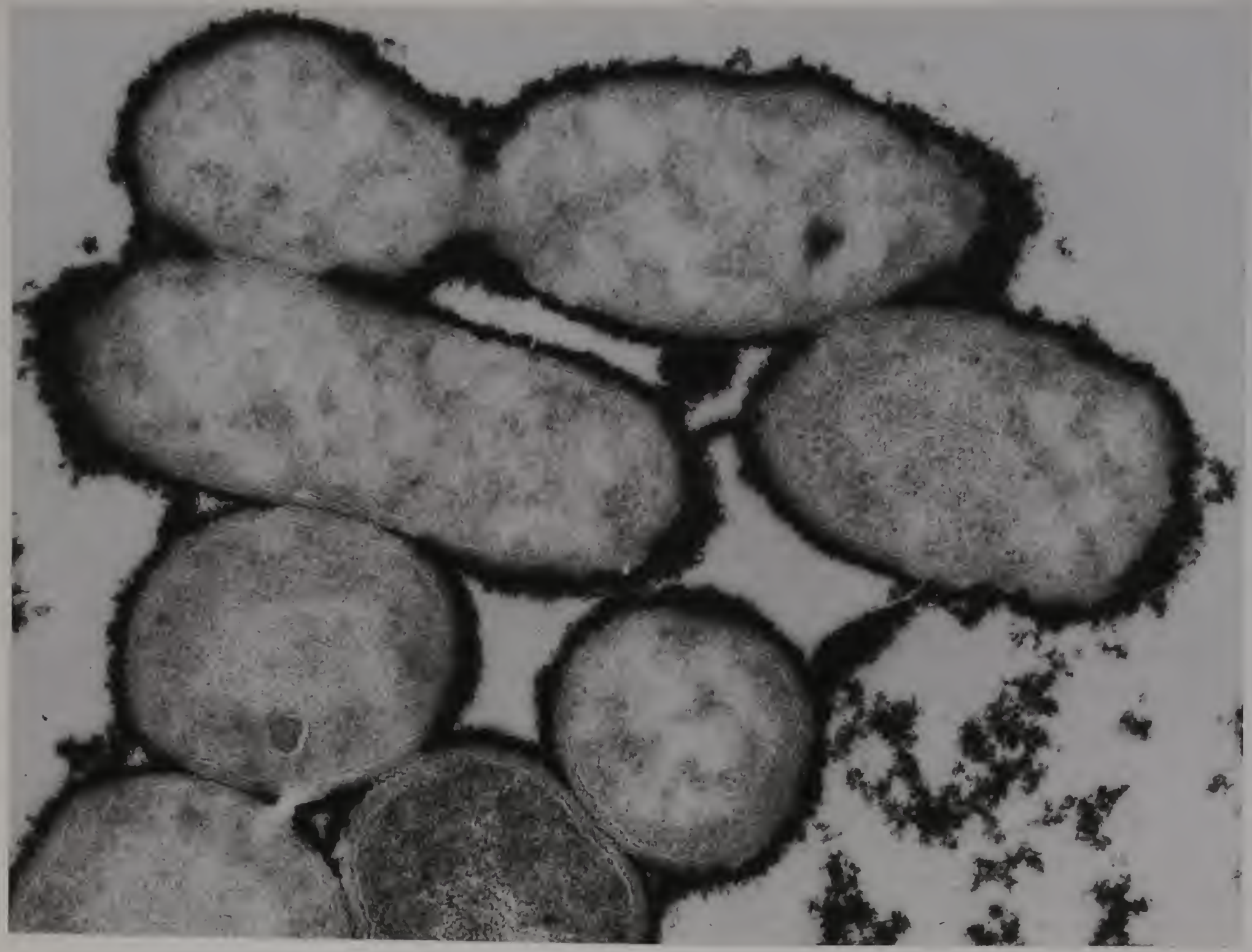


Fig. 26.--Electron micrographs of thin sections of *Mycobacterium phlei*

Diazinon-treated culture

Membrane thickened and in possible prelytic stage. Cells appear fused together. Black amorphous film may be artifact produced by Diazinon treatment.



Diazinon culture, and a greater proportion of degenerate cells than in the control (Fig. 27).

III. Disappearance of Diazinon in Bacterial Cultures, and Controls:

A. Analysis by Electron Capture Gas Chromatography:

Anaerobic cultures and sterile controls with the pH adjusted to the respective cultures achieved in 24 hours were supplemented with Diazinon to 30 ppm. They were sampled to determine if Diazinon were degraded, and to what extent. After 24 hours, the anaerobes produced an acidic environment in which the pH fell from neutrality to 4.1 in the various cultures. Cultures and parallel controls showed corresponding decreases in Diazinon concentration. However, less Diazinon was recovered from the anaerobes numbered 2, 7, 7B, and 4 than in their respective controls (Figs. 28, 29, 30). The least Diazinon was recovered in the cultures which produced the greatest acidity.

This would indicate that Diazinon was hydrolyzed by the acidic environment which was produced as a result of incomplete oxidation of organic matter by anaerobic metabolism.

Since pH played a primary role in Diazinon hydrolysis, the pH and Diazinon concentration were monitored in a culture of a facultative anaerobe #7 grown under various conditions. The organism produced changes in pH from neutrality to pH 6.8, 7.0, and 8.6, during anaerobic, semi-aerobic, and aerobic

Fig. 27.--Electron micrographs of negative stains of *R. rubrum* M_2B Diazinon-treated culture. Cell wall detached.

Fig. 28.--Comparison of Diazinon degradation in anaerobes 2, 7 and pH adjusted sterile controls.

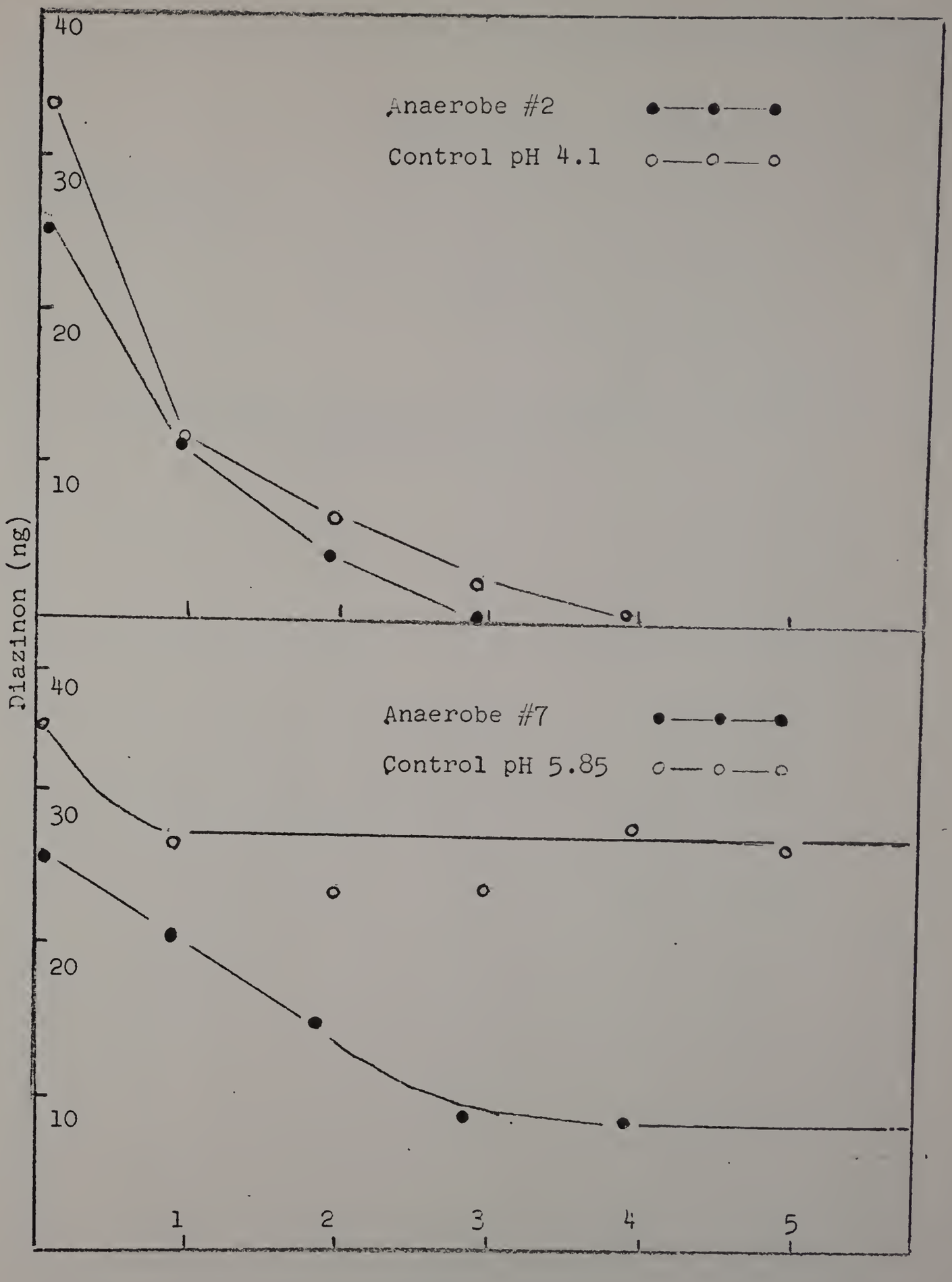

40
30
20
10
Anaerobe #2
Control pH 4.1
Diazinon (ng)
40
30
20
10
Anaerobe #7
Control pH 5.85
1
2
3
4
5
DAYS

Fig. 29.--Comparison of Diazinon degradation in anaerobes 4, 11 and pH adjusted sterile controls.

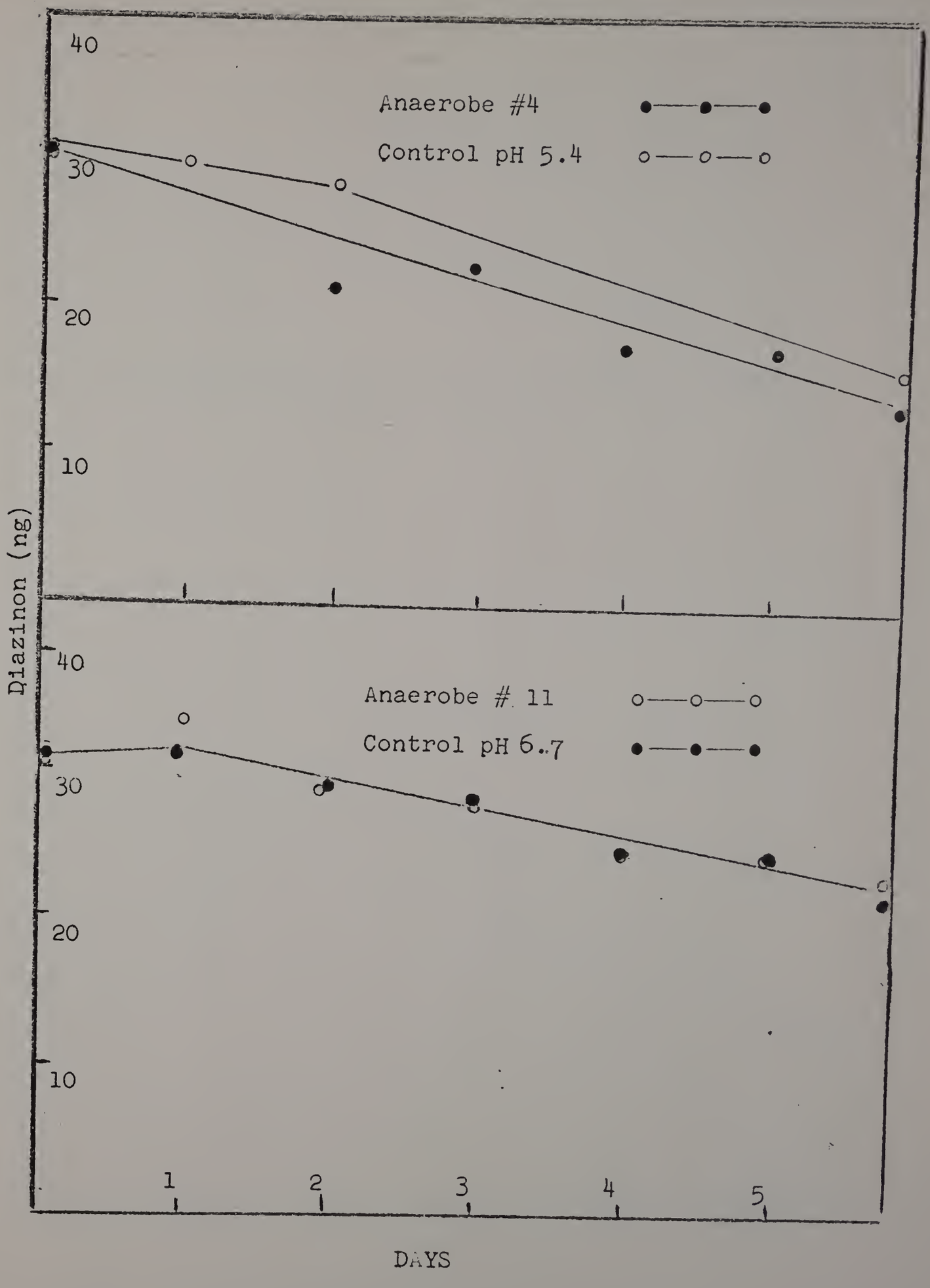
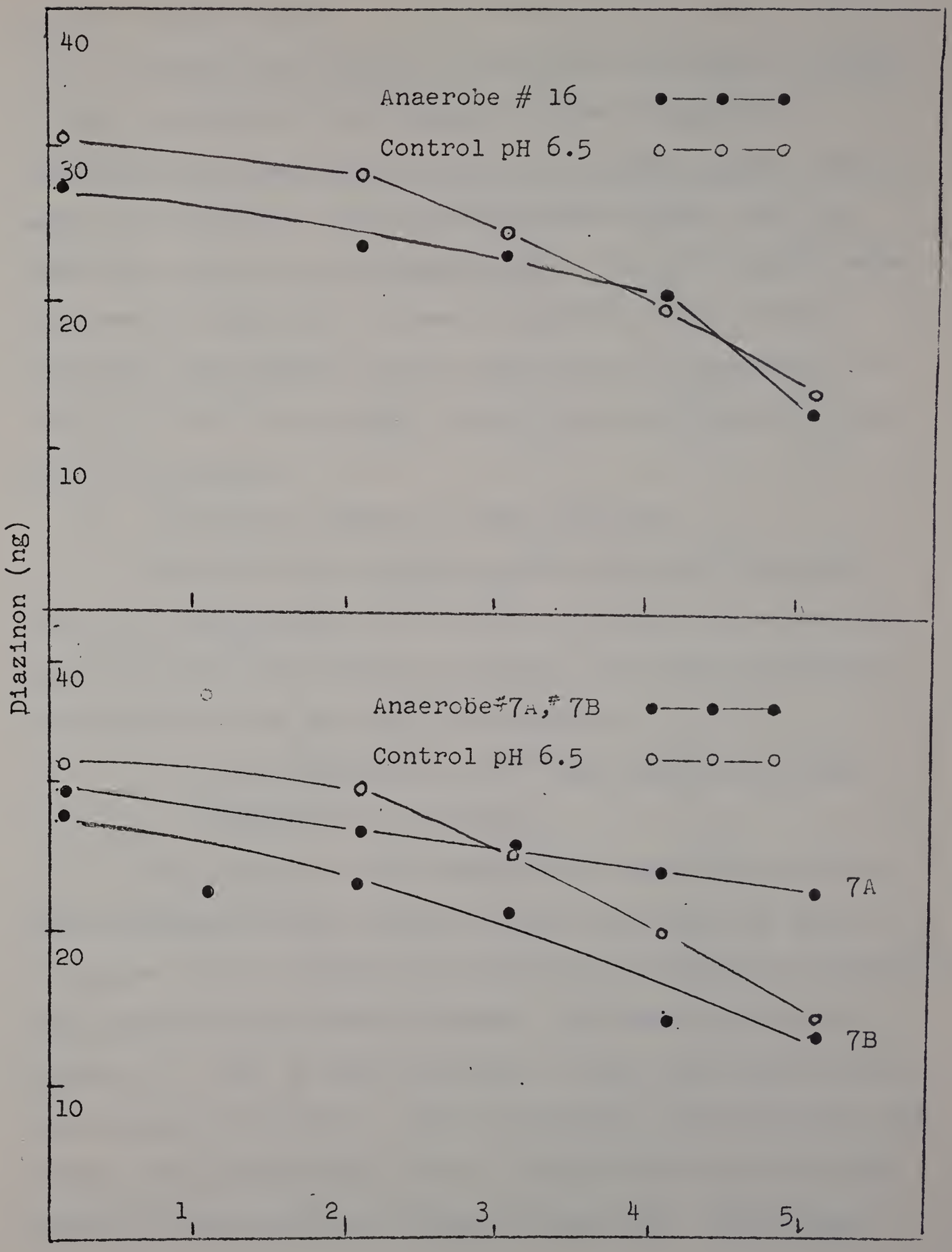

40
30
20
10
Anaerobe #4
Control pH 5.4
Diazinon (ng)
40
30
20
10
Anaerobe # 11
Control pH 6.7
1
2
3
4
5
DAYS

Fig. 30.--Comparison of Diazinon degradation in anaerobes 16, 7A, 7B, and pH adjusted sterile controls.


40
30
20
10
Anaerobe # 16
Control pH 6.5
Diazinon (ng)
40
20
10
Anaerobe #7A, #7B
Control pH 6.5
7A
7B
1
2
3
4
5
Days

growth, respectively.

The pH was directly correlated with oxygen tension in this culture, for the greatest percent degradation occurred in an anaerobic, acidic, environment (40%), the next in an aerobic, alkaline environment (27%), and the least in a neutral environment (24%). Similar results were produced in controls. It was of interest that a single organism, grown under varying conditions could generate an acidic or basic environment which caused hydrolysis of the Diazinon molecule.

B. Analysis by Carbon 14 Label Studies:

Since previous results by GLC analysis indicated Diazinon disappearance in bacterial cultures the extent of degradation of the breakdown products, and their possible incorporation into the cell was examined.

1. Use of Radioactive C^{14} Ring-labeled Diazinon and Unlabeled Diazinon:

The purpose of this preliminary experiment was to trace Diazinon transformation in the supernate and cells of a culture, and to examine its ability to oxidize the pyrimidine ring completely to carbon dioxide. Cultures 7, 7B and a mixture of 7 and 7B were incubated 5 days with ring-labeled and unlabeled Diazinon. After incubation, approximately 33% of the cold Diazinon and 11% of labeled Diazinon were converted to water soluble products (Table 14). Thin layer

TABLE 14.--Distribution of C^{14} of ring-labeled Diazinon and breakdown products in supernate, cells, and $C^{14}O_2$ of organisms 7 and 7B in anaerobic culture

Sample	Counts per Minute 7	7B	7-7B	Control
pH	5.85	6.6		5.85
Supernate: Fractions				
Petroleum Ether	1460	1906	1658	2213
Aqueous	221	255	150	295
Cells: Fraction				
Petroleum Ether	30	40	30	--
Filtrate (wash)	Total 227			
Petroleum Ether				
$C^{14}O_2$ as $BaC^{14}O_3$	2	2	2	--
Total Counts Recovered	1788	2278	1915	2508
% Diazinon Converted to Water Soluble Products	12%	11%	9%	11%
% Recovered Compared to a Control	71%	90%	76%	100%

chromatographs confirmed the presence of Diazinon in the petroleum ether extract of the supernate. No Diazinon was detected in the aqueous fraction of the supernate by thin layer chromatography, thus confirming that all the Diazinon was removed by the extraction procedure. The extract of the cells and filtrate contained approximately 1% and 3% of the labeled Diazinon, respectively, establishing that very little Diazinon had adhered to the washed cells. Ring-labeled Diazinon was not degraded to carbon dioxide in any culture, suggesting stability of the pyrimidine ring under these conditions.

2. Use of Ring-labeled and Side-chain-labeled Diazinon:

This experiment was designed to determine if either the ring or the side chain moiety were oxidized to carbon dioxide, and if Diazinon or a related compound were accumulating in the cells. An attempt was made to trace the label in the supernate, sonicated and whole cells, and in carbon dioxide released.

An anaerobe, 2, was incubated with ring and side chain-labeled Diazinon. After 5 days, ring and side chain-labeled cultures contained only 18% and 8%, respectively, of the parent compound. Approximately 87% of the parent compound had been converted into water-soluble components (Table 15). Comparison with controls indicated 100% of

TABLE 15.--Distribution of C^{14} ring-labeled and side chain-labeled Diazinon and breakdown products in supernatant, sonicated and whole cells, and CO_2

	Counts per Minute			
	Ring Labeled		Side Chain Labeled	
Sample	2	Control*	2	Control*
pH	4.1	5.4	4.1	5.4
Supernatant: Fractions				
Petroleum Ether	189	822	305	3477
Aqueous	1070	125	547	289
Whole Cells: Fractions				
Petroleum Ether	0		1	
Aqueous	6		3	
Sonicated Cells: Fractions				
Petroleum Ether	0		0	
Aqueous	2		3	
Filtrate (wash)				
Petroleum Ether	42		97	
CO_2 as $BaCO_3$	0		0	
Total Counts Recovered	1309	947	956	3766
% Diazinon Converted to Water Soluble Products	82%	13%	91%	8%
% Recovered Compared to a Control	100%		26%	

*Control pH 5.4 used to determine the approximate number of counts added. A lower pH would have resulted in unrecoverable counts of side chain label as ethanol.

the ring label, and only 25% of the side chain label was recovered. Neither culture oxidized the pyrimidine ring nor the ethoxy group in the side chain to labeled CO_2, suggesting stability of the moieties under these conditions. No counts were detectable in sonicated cells or whole cells after washing, but approximately 6% of the label appeared in the cell wash. These results indicated that a small amount of Diazinon had adhered to the cells, but was removed by washing, and also that no Diazinon or breakdown products had accumulated in the cells.

3. Use of Solvent Partition to Determine Distribution of the Label, and Identification of Products:

An experiment was designed to trace the C^{14} label as it appeared in various degradation products. A qualitative and quantitative evaluation of the parent Diazinon and breakdown products was made on the basis of their solubilities in benzene, butanol, diethyl ether and in the aqueous fractions. Comparison of the number of counts in each fraction with a sterile control for pH was the basis for determining the relative degradability of each compound.

Cultures which hydrolyzed Diazinon to various degrees were chosen (Table 16).

As in previous experiments, recovery of the ring-label was excellent, almost 100% for most cultures, but recovery

TABLE 16.--Detection and quantitative evaluation of Diazinon and breakdown products as traced with ring-labeled Diazinon

	Counts per Minute							
	Culture	Control	Culture	Control	Culture	Control	Culture	Control
	2		7		1		M_1	
pH	4.1	4.1	5.85	6.2	5.95	6.2	6.5	6.9
Benzene	7	215	241	552	451	552	526	573
Butanol	679	920	663	424	536	424	475	278
Ether	141	158	14	14	26	14	25	15
Aqueous	92	86	103	110	104	110	82	70
Total	919	1389	1021	1100	1117	1100	1107	936

of side chain label was poor. In cultures incubated with ring-labeled Diazinon, fewer counts appeared in the benzene fraction and conversely, more counts in the butanol fractions, than in the controls (Tables 16, 17). The cultures contained less Diazinon and more 2-isopropyl-4-methyl-6-hydroxypyrimidine than the controls. Again, the conversion of Diazinon to breakdown products increased with decreasing pH, and ranged from 92% at pH 4.1 to 52% at pH 6.5. The radioactivity in the diethyl ether layer increased with decreasing pH, and was highest at pH 4.1. Approximately 13% of the pyrimidine ring was cleaved in culture 2, and its control.

Equal amounts of Diazinon were present in ring and side chain-labeled cultures of 7 and 2 when compared to their controls (Table 18). Butanol extracts contained 64% and 74% of labeled 2-isopropyl-4-methyl-6-hydroxypyrimidine and 10% diethyl thiophosphoric acid in cultures 7 and 2. The ring moiety was quite stabile. The side chain, in contrast, readily degraded to ethanol, which volatilized when the samples were prepared for C^{14} counting. This problem was subsequently avoided by use of liquid scintillation counting.

Radioactivity increased in the aqueous layer with decreasing pH (Table 19). In most instances, the radioactivity in the samples was similar to the controls.

TABLE 17.--Detection and quantitative evaluation of Diazinon and breakdown products as traced with ring-labeled Diazinon

	% of Total Counts Recovered							
	Culture	Control	Culture	Control	Culture	Control	Culture	Control
	2		7		1		M_1	
pH	4.1	4.1	5.85	6.2	5.95	6.2	6.5	6.9
Benzene	0.8%	16%	24%	50%	40%	50%	47%	61%
Butanol	74%	66%	64%	39%	48%	39%	42%	29%
Ether	15%	12%	1%	1%	2%	1%	2%	2%
Aqueous	10%	6%	10%	10%	10%	10%	7.4%	8%
% of Diazinon Converted to other compounds:	92%	84%	77%	50%	60%	50%	52%	39%
% of label recovered compared to controls:	66%		92%		100%		100%	

TABLE 18.--A comparison of the degradation of ring and side chain-labeled Diazinon by organisms 7 and 2

Sample	7 Side-Chain	7 Ring	2 Side-Chain	2 Ring
Benzene	219	241	9	7
Butanol	80	667	65	679
Ether	0	14	2	141
Aqueous Layer	-	103	-	92
Total		911		919

TABLE 19.--Detection and quantitative evaluation of a breakdown product, ethanol, of side-chain labeled Diazinon in the aqueous fraction of the supernate of various anaerobic cultures

					Liquid Scintillation			
Controls:	pH	4.5	5.4	5.6	5.9	6.9		
CPM		990	800	800	1060	720		
Samples:	Number	2	3	1	7	8	11	M_1
	pH	4.1	5.9	5.95	5.95	6.1	6.9	6.5
CPM		1610	892	712	680	977	412	330

The lability of the ethoxy group in side chain-labeled Diazinon negated its use in tracing compounds in various layers (Table 20), but the compound was useful in determining the oxidation of side chain to carbon dioxide.

4. Conversion of Side-chain Moiety to Carbon Dioxide:

All of the anaerobes and 14 of the aerobes, including an Acetobacter sp., were incubated with side chain-labeled Diazinon to determine if any culture could oxidize the alpha carbon of the side chain to CO_2. No label was detected in the $BaCO_3$, signifying that none of the cultures was capable of converting the alpha carbon of the side chain to CO_2.

C. The Nature of Hydrolysis of Diazinon; Enzymatic, or Non-Enzymatic:

This experiment was designed to identify the hydrolysis products, and to investigate the origin of the hydrolysis as to its enzymatic, or non-enzymatic nature.

1. Use of Polyacryamide Gel Fractionation and Autoradiography:

Cells of culture 7 were sonicated, and the preparation was divided into 2 equal samples, A and B.

Sample A of the crude, sonicated cell preparation was incubated with ring-labeled Diazinon, and hydrolyzed 13% of the Diazinon.

TABLE 20.--Detection and quantitative evaluation of Diazinon and breakdown products as traced with side chain-labeled Diazinon in various anaerobic cultures

	Counts per Minute							
	Culture	Control	Culture	Control	Culture	Control	Culture	Control
	2		7		1		M_1	
pH	4.1	4.1	5.82	5.9	6.2	6.2	6.5	6.9
Benzene	9	26	219	43	36	58	100	469
Butanol	65	135	80	130	131	254	199	76
Ether	12	46	0	12	0	30	33	-
Total	86	207	299	185	167	342	311	545

Approximately 87% of the Diazinon was extracted from the crude, sonicated cell preparation, as compared to 98% from the control (Table 21). Autoradiography confirmed

TABLE 21.--Hydrolysis of ring-labeled Diazinon by a sonicated cell preparation of culture 7

	CPM	
Fraction	Sonicated Cells	Control, H_2O
Benzene	2703	4811
Butanol	515	123
Total	3218	4934
% Total as Diazinon	87%	98%

the presence of 2-isopropyl-4-methyl-6-hydroxypyrimidine in the sample, and its absence in the control (Table 22).

TABLE 22.--Extracts of a sonicated crude cell preparation of isolate 7 incubated with ring-labeled Diazinon

	Rf					
Solvent	Benzene		Butanol			
	Sample	Control	Sample	Control	Ring	Cells
TLC Chloroform Development	0.65	0.65	-	-		
Acetone-Butanol Development	-	0.50	0.35	-	0.42	0.4

In a separate experiment, boiled and non-boiled crude sonicated cell preparations were incubated with ring-labeled Diazinon, and exhibited the same degree of hydrolysis, confirming that the nature of hydrolysis in culture 7 was non-enzymatic (Table 23).

TABLE 23.--Hydrolysis of ring-labeled Diazinon by a sonicated cell preparation of culture 7: boiled, and non-boiled

	CPM		
	Sonicated Cells		Control, H_2O
	Boiled	Non-Boiled	
Benzene	315	315	472

Sample B was fractionated, and protein content in fractions 1 through 11 determined (Fig. 31). The fractions hydrolyzed ring-labeled Diazinon to varying degrees. Fraction 7 was divided into two equal portions, and one half was boiled vigorously to destroy the enzymes. Both portions caused 66% hydrolysis of the parent molecule, the highest of any of the fractions tested. Since boiled, and non-boiled portions produced the same degree of hydrolysis, the samples must have been responsible for non-enzymatic, rather than enzymatic degradation of Diazinon.

Since the polyacrylamide gel fraction 7, which caused the greatest hydrolysis of Diazinon, also had the highest absorbancy at 260 nm (Fig. 32), nucleic acids,

Fig. 31.--Protein and radioactive Diazinon content of fractions 1-11.

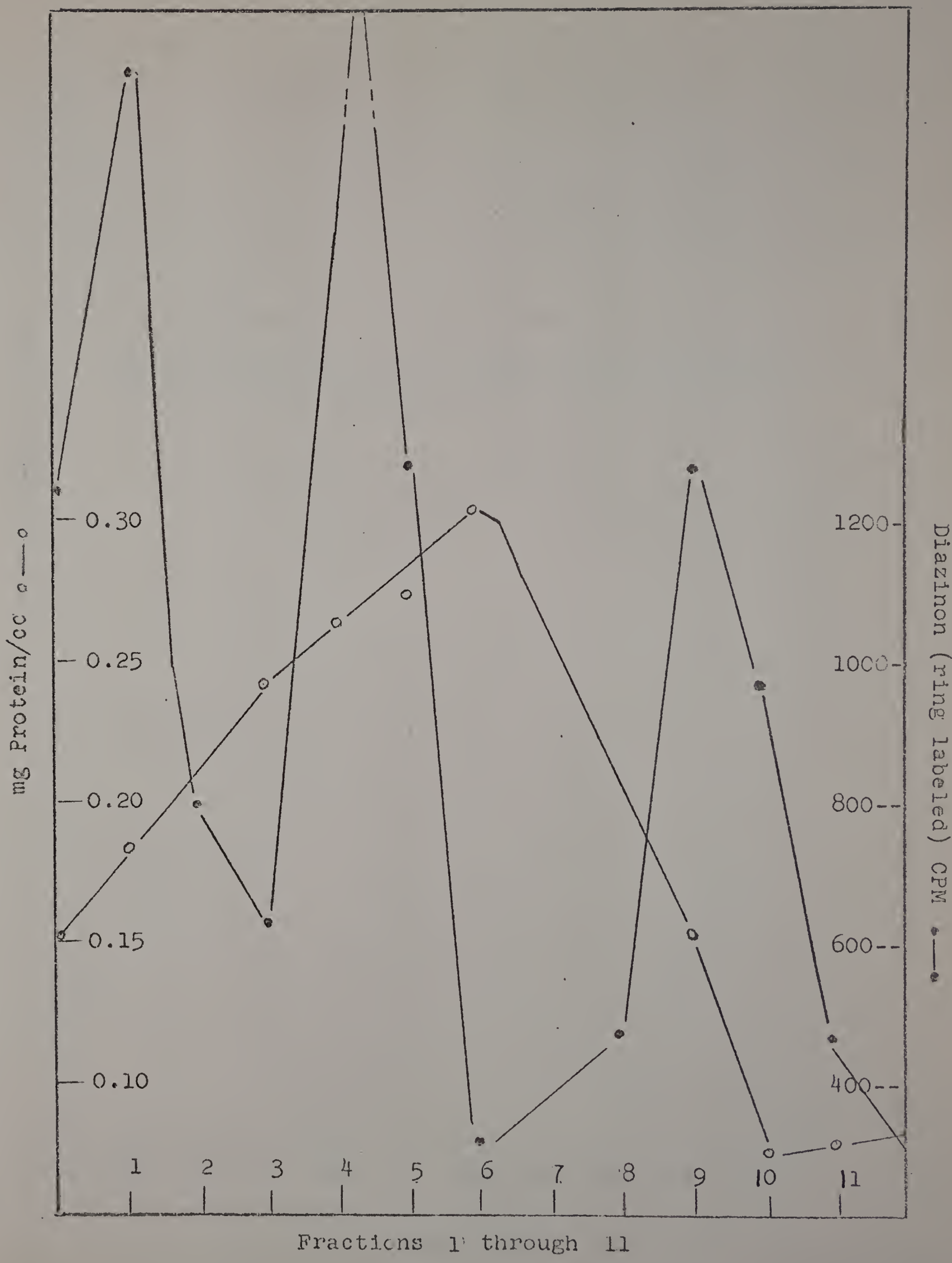
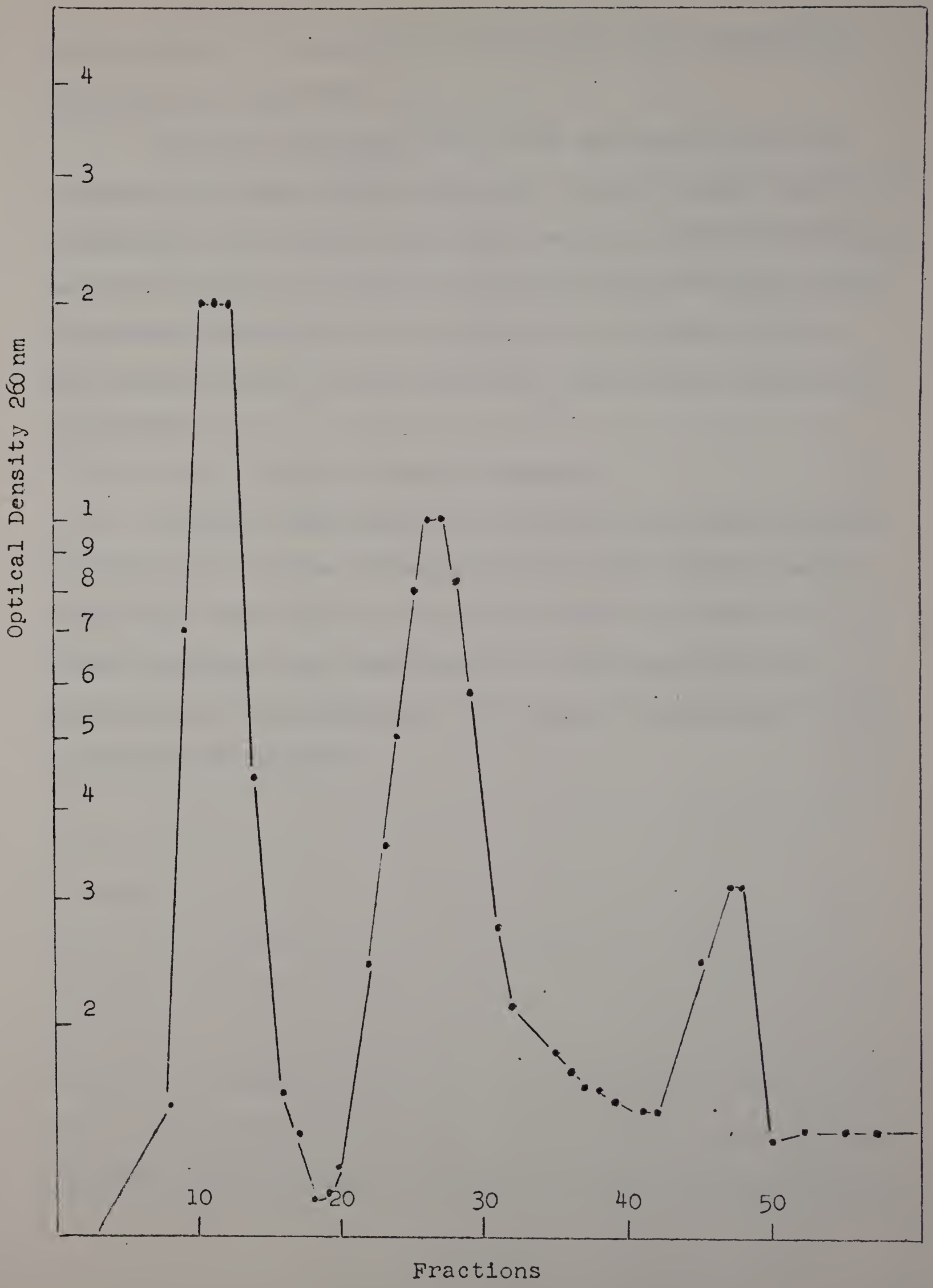

mg Protein/cc
0.30
0.25
0.20
0.15
0.10
1200
1000
800
600
400
Diazinon (ring labeled) CPM
1 2 3 4 5 6 7 8 9 10 11
Fractions 1 through 11

Fig. 32.--Optical density (260 nm) determinations of fractions 1-60 of a sonicated cell extract of culture 7.


Optical Density 260 nm
4
3
2
1
9
8
7
6
5
4
3
2
10
20
30
40
50
Fractions

amino acids, and adenosine triphosphate were tested for hydrolyzing properties.

Nucleic and amino acids were neutralized and incubated with ring-labeled Diazinon. In all cases, after incubation, the sample contained the same amount of Diazinon as the control. A mixture of ATP and Diazinon also failed to produce hydrolysis of Diazinon, as determined by GC, but caused a shift in the mixtures' ultraviolet absorption to 250 nm.

3. Use of Purified Enzymes:

Several approaches to the problem and the ultimate question of Diazinon biodegradability were tested simultaneously. The ability of isolated known esterases to cause hydrolysis was investigated. Alkaline and acid phospatases, phospholypase C, and deoxyribonuclease failed to hydrolyze Diazinon.

DISCUSSION

With the recalcitrance of many insecticides a matter of growing public and scientific concern, the biodegradability and ecological stresses induced by pesticides have become matters of urgent investigation. The organophosphates have latterly replaced the chlorinated hydrocarbons on the assumption of their relatively easy susceptibility to microbial attack. Detailed investigation, however, has shown that degradability in the case of Diazinon may not be biological in origin, and, more importantly, that the disappearance of the parent compound is not to be equated with innocuousness. A further light cast by this thesis is the role played by the neglected stratum of the soil biota, the facultatively anaerobic soil bacteria.

Initial investigations in selective anaerobic cultures showed that Diazinon was not only a nutritionally inaccessible carbon source, but an inhibitory agent as well. The inaccessibility of Diazinon as a nutritional source could have been anticipated from the sharply limited solubility of Diazinon in water, which, a priori, would render it practically inaccessible to microbial metabolism. Thus, it would appear that the population selective effect reported by Gunner and co-workers (16) is induced not only

by a nutritional advantage offered to a selected population, but also to their relative resistance to the toxic effect which diminished competing sensitive populations.

Diazinon, even in its low solubility of 40 ppm, was generally detrimental to bacterial growth. The nature of Diazinon restriction on bacterial development was a diffuse one. In certain instances, there appeared to be an enhanced lysis, or clumping, suggesting the interaction of Diazinon with the cell wall constituents. Also, cells failed to separate after division. A pinching occurred, but cells remained in chains. On occasion, the site of division appeared to be toward the end of the cell, rather than in the central area. Another cytological aberration which appeared consistently was the reduction of the number of flagella in one isolate. In general, then, the presence of Diazinon would appear to be inimical to bacterial growth in a diffusely disruptive pattern, probably, initially by interaction with the lipid constituent of the cell wall. One explicit pattern of toxicity which emerges from these studies was that exercised by the ethanol moiety of the Diazinon molecule. Under anaerobic conditions, ethanol may accumulate and at appropriate concentrations, cause a wide spectrum of damage to the cell, injuring the membrane, and denaturing enzyme systems. The close parallel of toxicity obtained with ethanol and the side chain of Diazinon would

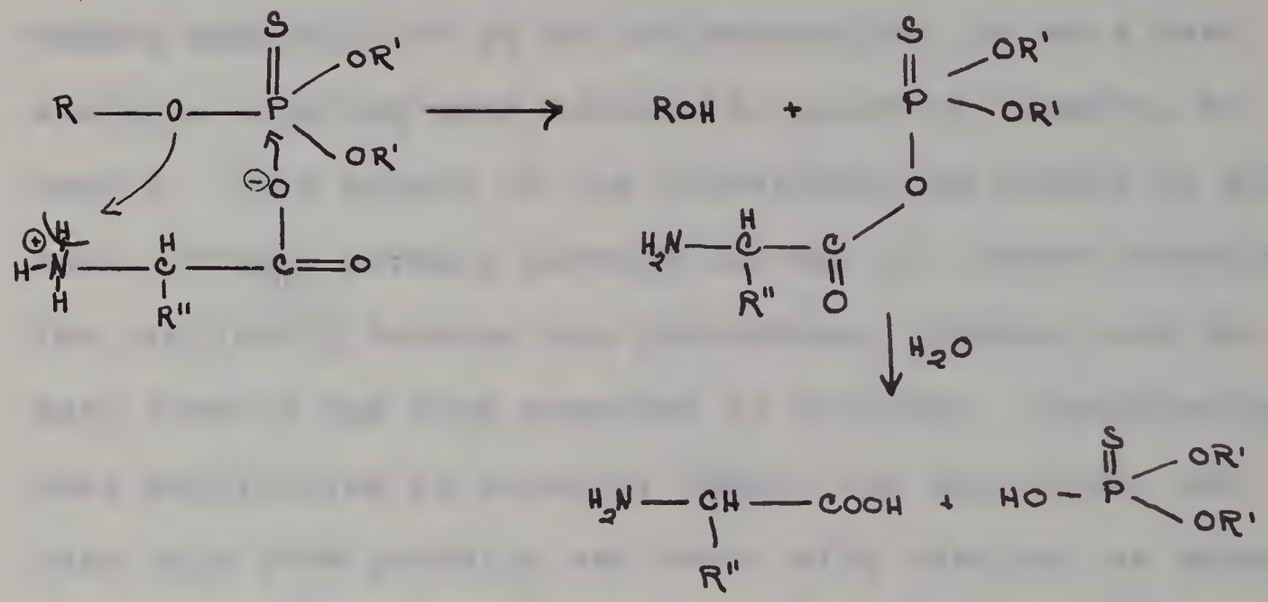
suggest that at least one source of Diazonon damage is its ethoxy group. In terms of population succession, the varying degrees of sensitivity shown by different microbial species to ethanol may be a factor in determining population response.

In a similar light, the lipid content of the cell wall may also be a measure of microbial response to the lipophilic nature of Diazinon in interacting with it to produce clumping, lysing and other degenerative changes.

From an ecological standpoint, it is of paramount importance to emphasize that population changes can be induced over a long period by the degradation products of even so-called degradable pesticides. Apart from the work of Gunner et al. (16) with bacteria, Malone and his co-workers (26) have shown that long-term changes have been induced with higher plants after Diazinon application, even following the disappearance of the parent compound. It, therefore, follows that increasing concern must be shown to the effect of pesticide residues, which though not identified, nonetheless are sharply expressed in the ecological stresses that they produce.

Diazinon, in an acidic environment, hydrolyzed into water-soluble components. This cleavage was catalyzed by an acidic pH produced by the anaerobes. The slightly higher rate of hydrolysis in old cultures as compared to

controls was probably catalyzed by amino acids (29) released from the lysing cells. A mechanism for amino acid catalyzed hydrolysis is as follows:



The ring compound, 2-isopropyl-4-methyl-6-hydroxy-pyrimidine, was very resistant to attack. It was designed specifically for stability because it was to act as a carrier for the active fraction of the pesticide, the side chain, specifically the P=S. The stability was attributed to the resonant nature of the ring, and the positions of the substitute groups. The original pyrimidine ring was substituted in the 2, 4 and 6 positions satisfying the imbalance produced by the strongly electrophilic nitrogens, and making "the ring system extra-ordinarily stable to

hydrolytic cleavage" (6).

The ring compound, 2-isopropyl-4-methyl-6-hydroxy-pyrimidine, though not an apparent determinant in population succession, may, if incorporated into the cell in minute amounts, act as an antimetabolide, or as a base analogue coupling with adinine in place of thymine, or uracil. This aspect of the investigations should be pursued further, perhaps through the use of tracer techniques. The similarity between the pyrimidine, thymine, and the keto form of the ring compound is striking. Considering that equilibrium is strongly toward the keto form, and that this form probably can react with adenine, as shown by a shift in the ultraviolet absorption spectra, a possibility exists that this compound may be an antimetabolite, and may have potentially mutagenic properties.

Naturally occurring pyrimidines can be degraded by a few bacteria, and some yeasts (7, 17, 25, 33, 34). The ability to degrade a given pyrimidine is highly specific, and the saturation of one double bond, or the substitution of one group, or type of group for the original can yield a nondegradable molecule (34). Generally, compounds substituted in the 6 position, are very slightly oxidized, or not oxidized at all (34).

The data presented herein indicate Diazinon's hydrolysis products 2-isopropyl-4-methyl-6-hydroxypyrimidine,

diethyl thiophosphoric acid and ethanol are essentially recalcitrant under our anaerobic test conditions. These results are in agreement with those of Sethunathan and Yoshida, who studied the persistence of Diazinon in submerged soil (31).

APPENDIX

I. Media

A. Modified Morris Medium

KNO_3	0.2%
$MgSO_4$	0.02%
$CaCl_2$	0.001%
K_2HPO_4	0.7%
KH_2PO_4	0.32%
$FeCl_3$	0.001%
Glucose	1.5%
H_3BO_3	0.056 mg %
$MuCl_2 \cdot 4H_2O$	0.037 mg %
$CuSO_4 \cdot 5H_2O$	0.004 mg %
$NaMoO_4 \cdot 2H_2O$	0.015 mg %
$CoCl_2 \cdot 6H_2O$	0.0074 mg %
$ZnSO_4 \cdot 2H_2O$	0.005 mg %

B. Nutrient Agar

Yeast Extract (Difco)	0.5%
Casamino acids (Difco)	0.3%
Agar (Fisher Scientific Co.)	1.6%

C. Ethanol Agar

Yeast Extract	1%
Agar	2%

$CaCO_3$	1%
Ethanol (95%)	1.5%
D. EMB Agar	3.7%
E. Bacto-Gelatin	12%
F. Bacto-Litmus milk	10%
G. Triple Sugar Iron Agar	6.5%
H. Amino Acids:	Gly, Ala, Val, Lev, Ilev, Phe, Ser, Thr, Lys, Arg, Asp, Glu, CySH, CyS-CyS, Met, Tyr, Pro, Hypro, Try, His

II. Stains

A. Flagella: Bailey method modified by Fisher and Cohn.

1. Solution A

Tannic Acid	10%
$FeCl_3 \cdot 6H_2O$	6%
Filter	

2. Solution B

Solution A	3.5 ml
Basic Fuchsin .5% in 95% ethanol	.5 ml
Concentrated HCL	.5 ml
Formalin	2.0 ml
Filter	

Acid-wash clean, new slides. Rinse in H_2O, and

redistilled 95% ethanol. Flame slides. Suspend organisms (loopful) in 2-3 ml sterile, distilled H_2O 2 hours. Transfer 1 drop of suspension to slide. Tilt slide and allow fluid to flow to one end. Air dry.

Add solution A to slide 3.5 minutes. Pour off.

Add solution B to slide 7.0 minutes. Rinse with distilled H_2O.

Immediately cover with Diehl's carbol-fuchsin. Heat 1 minute on steam bath, rinse. Air dry.

Flagella appeared, and cells blue.

B. Capsule: Slide flooded with India ink.

Capsulated organisms appear as clear areas.

C. Phosphotungstic acid stain: Cells suspended in a 1% solution of phosphotungstic acid, and allowed to air dry on a grid before electron microscopic examination.

D. Ammonium Palladium Chloride for P=S Detection

0.05 g ammonium palladium chloride

0.2 ml concentrated HCl

9.8 ml H_2O

Spray on thin layer plate: Yellow spot is positive and specific for P=S.

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Microbial Interaction with Diazinon,
an Organophosphate Pesticide

Phyllis Joan Kuhn, B.S., University of Bridgeport
M.S., University of Massachusetts
Directed by: Dr. H. B. Gunner

ABSTRACT

Diazinon was essentially a nutritionally inert carbon source. It was hydrolyzed to ring and side chain by acid produced during anaerobic metabolism. The ring is degraded only when the pH is below 4.1. There is always slightly less Diazinon, and more hydroxypyrimidine in sample than in controls. Amino acids released by the bacteria may catalyze hydrolysis.

The enzymes, acid phosphatase, alkaline phosphatase, DNase and phospholypase C produced no hydrolysis of Diazinon.

Cultures (anaerobes and aerobes) are unaffected by Diazinon when cultured in enriched medium of yeast extract and casamino acids. Most exhibit a decrease in growth rate when cultured in Morris medium supplemented with only 0.025% yeast extract and Diazinon, as compared to controls. In anaerobic cultures, parent Diazinon, side chain, or EtOH,

but not ring compound, act as population determinants. Equivalent amounts of EtOH or side chain had equivalent effects on growth rates. In some cultures, the growth rate was decreased, and in one, unchanged. Total nucleic acid was decreased in some cultures, but this was a reflection of a decrease in the total number of cells.

Anaerobes treated with Diazinon clump, lyse, become less motile, exhibit partial degradation of the cell wall, or fail to separate after division, depending on the strain. These differences are subtle in 24 hr cultures, but become more apparent in older cultures as compared to controls. Phase contrast micrographs, and negative staining show these differences. Other data using tagged Diazinon, and thin layer chromatography, also indicate Diazinon is on the cell, rather than in the cell, for the strains tested.